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BIOLOGICAL BULLETIN

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THE
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THE EXTERNAL DEVELOPMENT OF THE BANDED DOGFISH OR POFADDERHAAI HAPLOBLEPHARUS EDWARDSII (M. & H.)

CECIL VON BONDE

Government Marine Biologist and Director of Fisheries, Union of South Africa

INTRODUCTION

This oviparous species of dogfish, previously known as *Scylliorhinus edwardsii* (Cuv.), is endemic to South African waters and has been recorded from Saldanha Bay, Table Bay, False Bay and the Agulhas Bank. The largest one on record measured 520 mm. This dogfish is fairly plentiful and specimens are on exhibition in the Sea Point Aquarium.

On November 5, 1942, a female was observed to lay two egg cases in one of the tanks of the Aquarium. One case was immediately removed and dissected to expose the egg, whilst the other was left in the tank as a control in the determination of the duration of incubation.

The egg was completely removed from the egg case and placed under observation in running sea water.

THE FEMALE REPRODUCTIVE ORGANS

The only external sexual characters in the female are the pair of cloacal papillae (Plate I, c.p.), one situated on either side of the median line immediately posterior to the cloacal aperture. The papillae themselves are perforate, and the abdominal pore situated in the center of each papilla connects direct with the coelom. The actual function of these abdominal pores is not known and it is obvious that, in view of the highly specialized structure of the female genital system, they have lost the function of acting as apertures through which the ova leave the body.

A dissection (Plate I) revealed the fact that there was only a single median ovary (ov.) present which contained ova in various stages of development. The ovary is situated in the middle portion of the coelom and is relatively large. The paired oviducts are highly specialized both in structure and function. The oviducts meet medially in the anterior part of the coelom (f.t.o.). Each oviduct may be divided into four distinct portions as follows:

(1) The fallopian tube (Plates I and II, f.t.) is extremely narrow and thin-walled and is about a quarter of the length of the whole oviduct. Its internal surface is slightly convoluted longitudinally. This portion is followed by (2) the albumen gland (a.g.) which is about one-eighth of the total length of the oviduct and is very thick-walled and muscular. This gland secretes the albumen which sur-

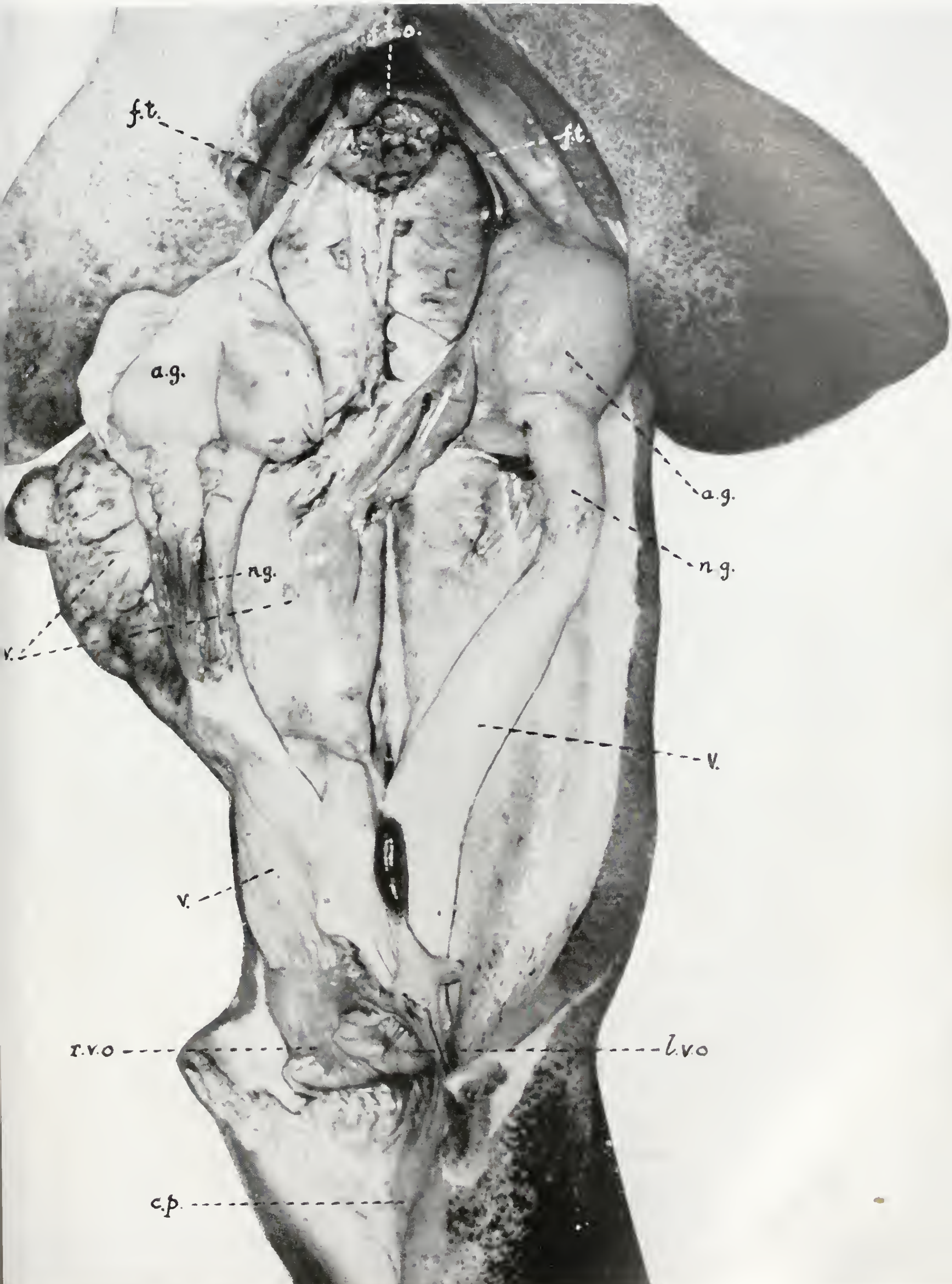
rounds the ovum, hence its internal surface is richly supplied with glands for this purpose. A transverse section of the albumen gland (Plate II, Fig. 3, l.a.g.) shows that the lumen is extremely narrow, the walls of the gland being completely apposed. Smith (1942, p. 703) in describing Bashford Dean's figure of the reproductive organs of an adult female *Heterodontus japonicus* states—"This drawing (my Text-figure 35) is not labelled, nor is it described in Dean's notes, and in the absence of the dissection some features are obscure. In the mid-line near the top of the figure, one readily notes the common abdominal opening of the oviducts. On the extreme right side of the figure the oviduct with its three divisions—oviduct proper, shell gland, and uterine portion—are easily identified." The part of the oviduct designated "shell gland" in the above quotation is very similar in external appearance to that of the present species, but as previously stated, this swollen portion functions as the albumen gland in the present species. There are no indications of a laminated structure in this gland as described by Gudger (1940, p. 550) for the nidamental gland of Chlamydoselachus. Immediately following the albumen gland is (3) the nidamental gland (n.g.) which is also about one-eighth of the total length of the oviduct and secretes the shell around the ovum. The interior surface of the nidamental gland is extremely well convoluted and is of a dark yellowish color. Widakowich (1907, p. 527) states—"Das Nidamentalorgan von Acanthias besteht aus einem cranial gelegenen Teile, der, wenn man nach Analogie mit Scyllium schliessen darf, Eiweiss erzeugt, aus einem mittleren, der, wie sich zeigen lässt, die Schale liefert, und aus einem untersten, wohl Schleim produzierenden Abschnitte." As the functions of the various parts of the oviduct of the present species, however, are distinctly diverse, it appears to be better to look upon each part as an independently functioning entity and parts two and three as here described may be looked upon as analogous to the cranial portion, and the central and caudal portions of the nidamental gland, respectively, as described by Widakowich. The last part (4) of the oviduct is the vagina (v.), occupying about one-half of the total length. This portion of the oviduct is designated the vagina in analogy to the use of this term in mammals in view of the fact that the intromittent organs (claspers) of the male are inserted through the vaginal orifices during copulation.

Each oviduct opens separately by a vaginal orifice (Plate II, l.v.o. and r.v.o.) into the cloaca. The vagina is well convoluted longitudinally to permit of distension both during copulation and during ovulation. In certain of the females dissected, parts of the mermaid's purse were present in the upper half of the vagina where it leaves the nidamental gland. These parts consisted principally of the anterior and posterior parts of the mermaid's purse complete with the tendrils (see Plate IV, Fig. 6).

PLATE I

Dissection showing the female reproductive organs of *Haploblepharus edwardssi* (M. & H.).
a.g., albumen gland. c.p., cloacal papilla. e.g., external gill-filaments. f.t., fallopian tube. f.t.o., coelomic orifice of same. h., hooks of clasper. h′., hooks normally covered by rhipidion. hy., hypopyle. l.a.g., lumen of albumen gland. l.v.o., left vaginal orifice. n.g., nidamental gland. ov., ovary. r., rhipidion. r.v.o., right vaginal orifice (opened). u., umbilicus with remains of cord. u.c., umbilical cord. v., vagina. v.b.v., vitelline blood vessel. y.s., yolk sac.

PLATE I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f.t.
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a.g.
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v.
V.
v.
r.v.o
l.v.o
c.p.

THE MALE REPRODUCTIVE ORGANS

The morphology of the male reproductive system follows the general plan of all Elasmobranchs. The testes are paired and occupy the anterior half of the coelom. From the anterior end of each testis the vasa efferentia lead to the epididymus which is much coiled. The epididymus eventually widens out into the vas deferens which expands and joins its fellow in the median line posteriorly, opening into the cloaca by a single pore.

The secondary sexual characters of this species are very distinct and it is necessary to describe them in more detail. As in all male Elasmobranchs, the basal element of each pelvic fin (*basipterygium*) is prolonged to form a stout, backwardly-directed rod which is sharply demarcated from the remainder of the fin and specially modified to form an intromittent organ commonly known as the clasper or myxopterygium. In this species the clasper is a very highly differentiated organ which differs in many respects from that found in other Elasmobranchs. In transverse section the clasper is almost completely round in its proximal half, the internal cartilagenous skeleton forming a tube which is almost completely filled with a thick muscular substance through the center of which passes a duct. In the distal part the skeleton only occupies the ventral portion of the clasper, being continued right to the tip. The latero-dorsal wings of this portion of the clasper are formed of thickened skin, the outer wing being folded over the curved inner wing so as to form a canal which is a continuation of the duct in the proximal portion previously mentioned. This canal opens on the dorsal side of the posterior end of the clasper in the form of a hypopyle (Plate III, Fig. 4, hy.). Schmidt (1930) first noted the existence of hooks in the clasper of *Halaelurus torazama* which is a genus closely related to the present one and he noted that these hooks were approximately 100 in number. The clasper of the present species also shows approximately 100 hooks on the outer lateral wall of the clasper (h. and Fig. 5).

Schmidt states—"Considering now what purpose this arrangement can serve, one comes to the conviction that the hooks can be used only for fastening the clasper on the wall of the vaginal part of the oviduct of the female during the copulation. Probably, when the clasper is introduced, the borders of its gutter are turned out and all the hundred hooks of the row are imbedded in the oviducal wall, holding the clasper as anchors. If the other clasper is introduced simultaneously in the other oviduct, the male is held by 200 little anchors. This arrangement may be necessary, as the female of this shark is larger than the male, which is perhaps carried about by the female during a copulation that may continue for a very long time.

"This arrangement of the organs of copulation of the male seems to be unique not only among the fishes, but perhaps in the whole animal kingdom," but he makes no mention of the other series of hooks (h'.) which lie on the inner lateral wall of the clasper and which are covered by a rhipidion (r.), whose function is to spray the spermatozoa in all directions in a radiating manner, under the pressure exerted

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PLATE II

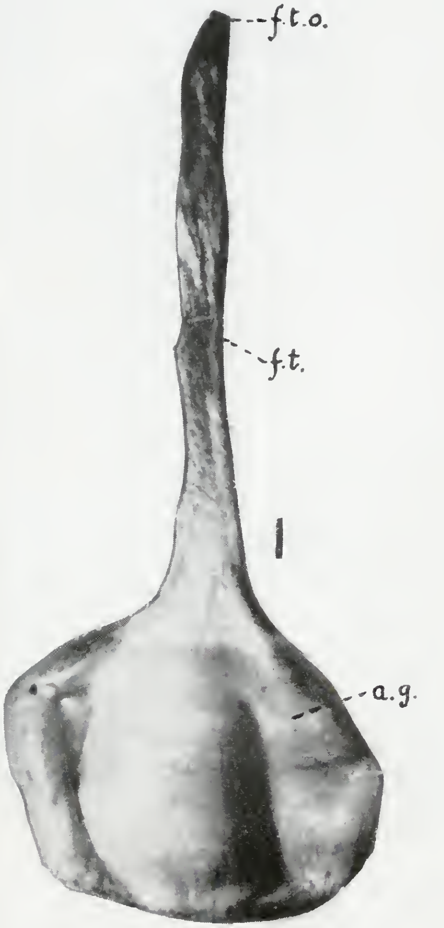
The left oviduct opened medially to show the internal structure.

FIGURE 1. Longitudinal section through the fallopian tube and the albumen gland.

FIGURE 2. Longitudinal section through the nidamental gland and the vagina.

FIGURE 3. Sagittal section through the albumen gland.

Plate II



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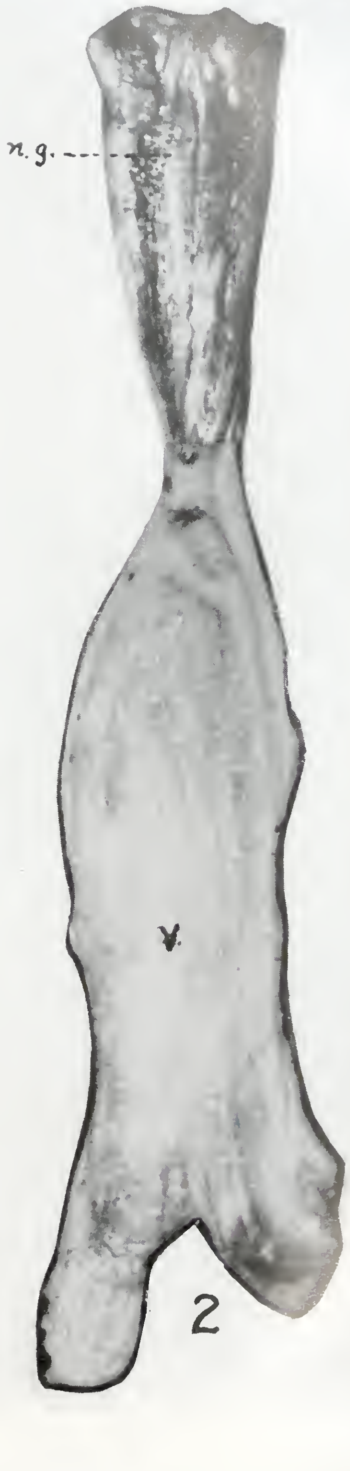

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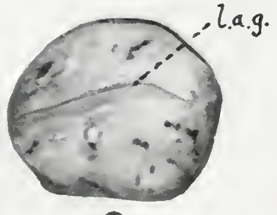

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by the water forcibly ejected by the siphon. The reason for this is obvious since fertilization takes place in the fallopian tubes and the spermatozoa, although motile, have to traverse the complete length of the vagina, nidamental gland, and the albumen gland to reach the ova in the anterior end of the fallopian tubes.

Leigh-Sharpe (1920) in describing the secondary sexual characters of *Scyllium catulus* mentions the presence of a siphon on the ventral surface of each pelvic fin. The present species also has such a siphon developed in the same position, although it is probably more analogous to the clasper gland mentioned by Leigh-Sharpe (op. cit., p. 260) than to the siphon. The whole of the clasper except the distal end is covered with dermal denticles. A deep dissection of the clasper showed that it is in no way innervated.

Development

In order to study the external development of this species a mermaid's purse was opened, the ovum removed from the purse and placed in a dish of running sea water. The albumen was completely removed so as to enable one to observe the development of the embryo.

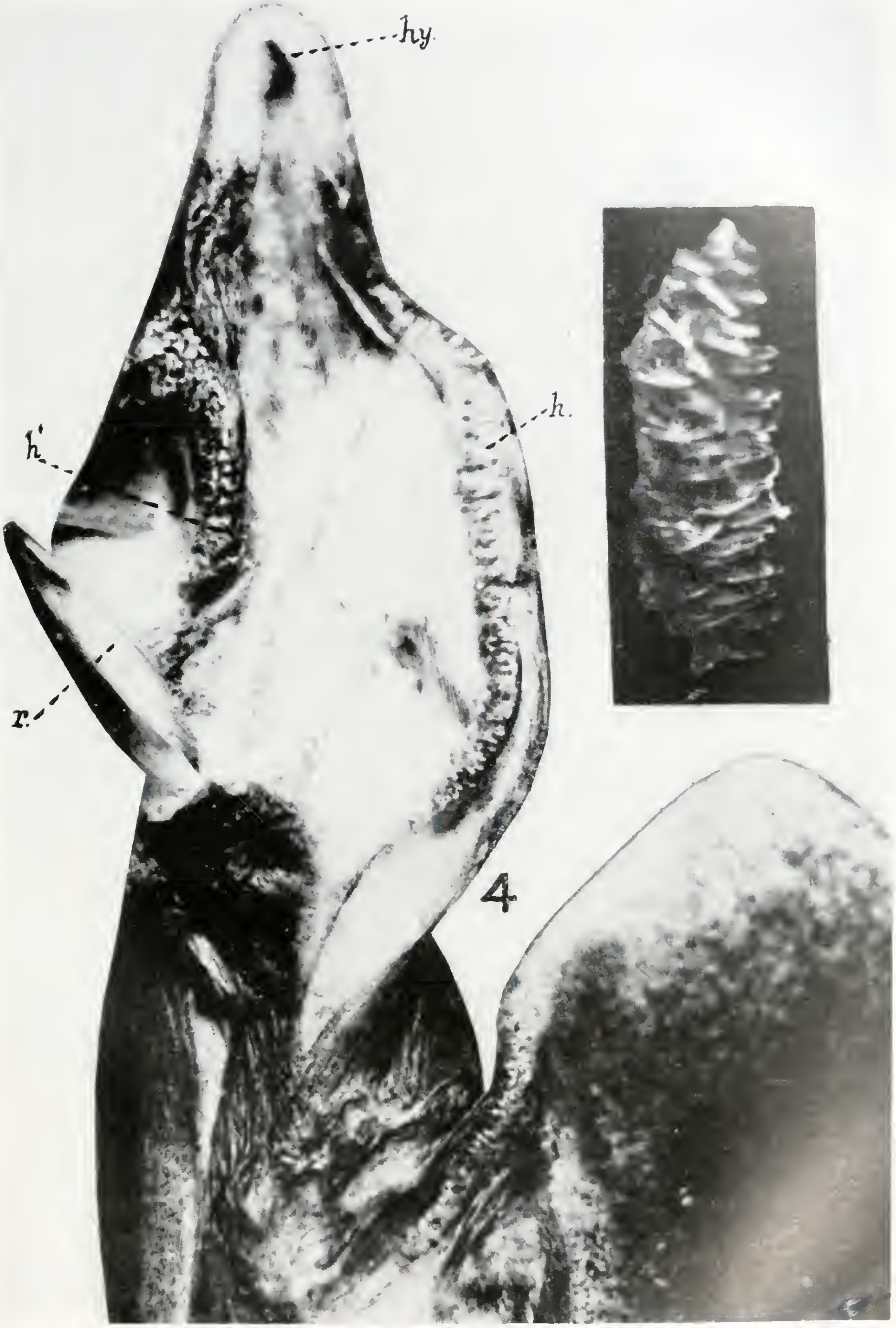
The egg case or mermaid's purse (Plate IV, Fig. 6) is more or less typical of those formed by oviparous dogfishes. The anterior end is broad and almost as wide as the main body and has a straight edge where the two halves have partially coalesced. From each lateral end a long coiled filamentous process, which gradually becomes thinner towards the distal end, arises. Near the base of each of these tendrils the purse has a longitudinal slit-like aperture (s.) which leads to the interior. These apertures only appear on one surface of the egg case. Posteriorly the egg case tapers until it is little more than half as wide as the anterior end. At the lateral extremities of this sharper end two processes arise which are the bases of the tendrils. These posterior tendrils are much longer and thicker than the anterior ones at their points of origin, but, like the latter, they also become thinner towards their distal ends. It is this pointed end of the purse which first appears through the vaginal orifice when the egg case is laid. The function of the tendrils is to anchor the egg case securely to rocks or seaweed to prevent its being buffeted about by the waves. The egg case itself is formed from keratin secreted by the nidamental gland. Widakowich (1906) stated that the mermaid's purse of *Scyllium canicula* is formed by a large number of separate elements ("Platten") which later, on exposure to sea water, adhere closely to form a complete shell (see also Hobson (1930, p. 580)), but in *Haploblepharus edwardsii* there is no evidence of such a formation of the egg case which is here laid down completely in two halves, the dorsal and ventral parts, the edges of which coalesce.

A dissection of the mermaid's purse shows that it is a structure formed of two portions which may be designated the dorsal and ventral halves, each half being hollow, the edges being apposed and completely fused along their whole length except at the blunt anterior end where the apposition is purely temporary. It is

Plate III

Figure 4. Dorsal view of the left clasper with lateral wings of distal portion distended to show internal structure. × 4.

Figure 5. A series of 40 hooks removed from the clasper. × 10.


hy.
h.
h'
r.
4

through this anterior end that the fish eventually escapes, hence the lack of fusion of these edges. The swollen central portion occupying about two-thirds of the internal space of the egg case, is filled by the relatively enormous yolk. Surrounding the yolk and occupying the remaining space of the egg case is the thick viscid transparent albumen. From the fact that during the artificial rearing of the embryo away from the egg case all the albumen was removed from the yolk, it is obvious that this albumen plays no part in the development or nourishment of the embryo at any stage, and purely serves the purpose of a protective cover around the embryo during its development. Its function could be regarded more or less as that of a shock absorber analogous to the function of the amniotic fluid in higher vertebrates.

The egg itself is between 30 and 35 mm. in diameter and is typical of the majority of Selachian eggs in regard to the distribution of yolk, being telolecithal. On one surface of the egg, which may be looked upon as the dorsal aspect, lies the germinal disc which is slightly lighter in color than the rest of the egg, has a diameter of 1.2 mm., and contains practically no yolk. Segmentation, as in all telolecithal eggs, is meroblastic since the cleavage planes are only restricted to the germinal disc and do not pass through the whole egg. The segmentation of the germinal disc is very similar to that of other Selachians and closely approximates that of the developing chick. The various later stages of gastrulation with the subsequent origin of the germinal layers is also typical of the class and it is not necessary to elaborate this feature. The folding off of the embryo takes place very early during development, at about 10 days after the egg is laid, the embryo then being about 2 mm. in length and attached to the yolk sac by a very well developed umbilical cord. The vitelline blood vessels at this stage are extremely prominent and spread out over the whole surface of the yolk sac. These vessels or capillaries unite into two median vessels which pass almost completely round the equator of the yolk (Plate IV, Fig. 9) to a region which lies distal from the umbilical cord. Here these vessels break up into two branches running at right angles to the equatorial vessels. These equatorial vessels pass along the sides of the umbilical cord and enter the embryo's heart.

The vitelline circulation is also typically similar to that appearing in the majority of oviparous Elasmobranchs and closely approximates that of the developing chick embryo. The umbilical cord is solid and the vitelline circulation plays a very important rôle in the transference of the nutritive food material from the yolk sac to the developing embryo. The absorption of the yolk material progressively increases with the growth of the embryo, with a concomitant decrease in the size of the yolk sac (cf., Plate IV, Figs. 7, 8 and 9). At an early stage in the development, the external gills, typical of most Selachian embryos, make their appearance

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PLATE IV

Stages in the external development of *H. edwardsii.*

FIGURE 6. The egg case or mermaid's purse, natural size.

FIGURE 7. A mermaid's purse opened to show a developing embryo 25 days old, natural size.

FIGURE 8. A 30-day old embryo. × 1.5.

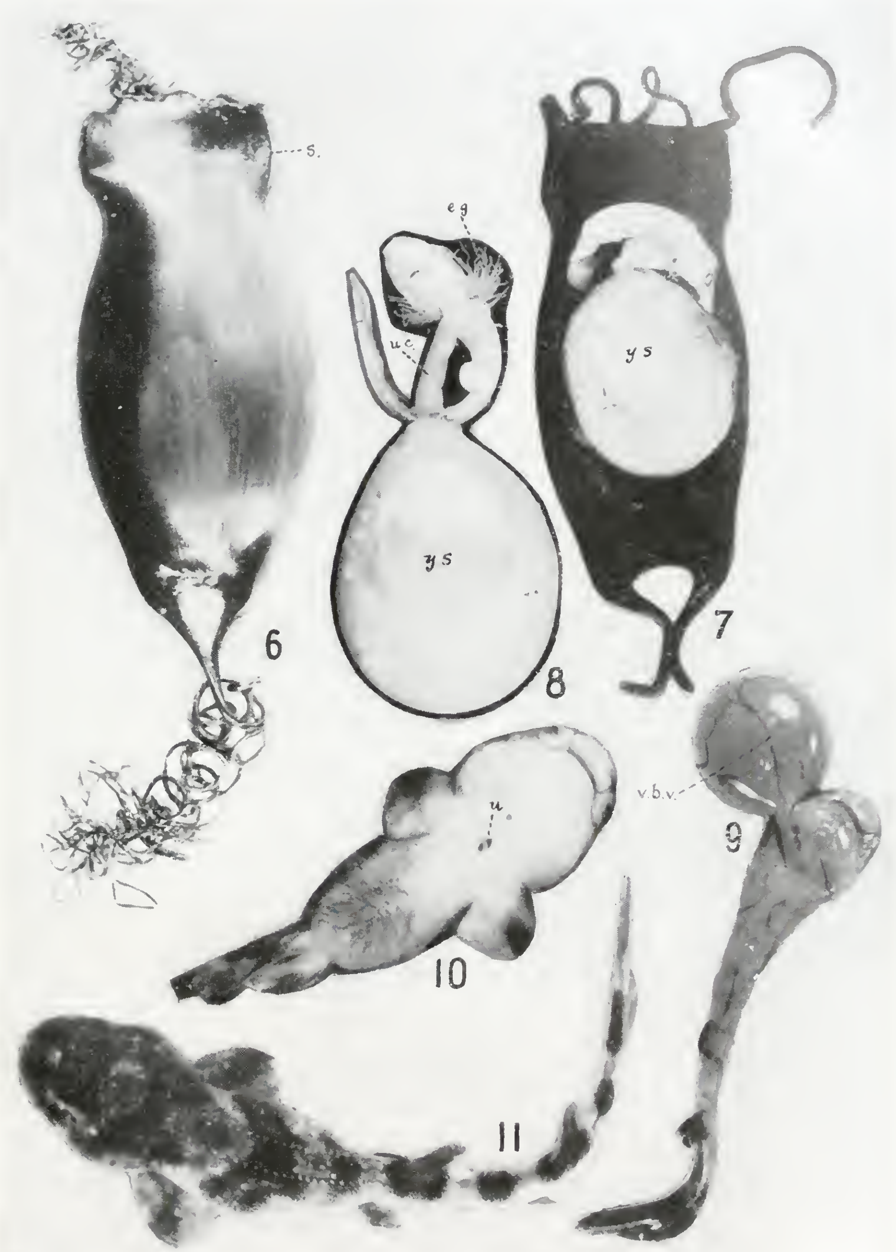
FIGURE 9. A 50-day old embryo. × 1.5.

FIGURE 10. Ventral view of embryo at time of hatching (104 days old). × 1.5.

FIGURE 11. Dorsal view of same.

All Photographs by Author.

PLATE IV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(Figs. 7 and 8). In origin these external gill filaments are totally unlike the external gills found in Crossopterygii, Dipnoi, and any Amphibia, and over their function and structure many zoologists have been puzzled.

Graham Kerr (1919, p. 157) states—"While external gills occur within three main sub-divisions of the vertebrates, namely, Teleostomatous fishes (Crossopterygians—the most archaic of existing Teleostomes), Lung-fishes, and Amphibians there are two main groups—Elasmobranchs and Amniotes—in which they are conspicuous by their absence. . . . As it happens, however, there is in the two groups mentioned a definite cause which seems quite competent to account for disappearance of external gills, namely, the development of a new organ—a yolk sac with its highly developed vitelline net work of blood vessels—which in addition to its primitive function must necessarily also function as a very efficient organ of respiratory exchange and so render any pre-existing respiratory organ no longer necessary."

In the present species these external gill filaments are richly supplied with blood vessels and in view of the fact that the embryo studied underwent its complete development in sea water outside of the mermaid's purse it is obvious that their primary function must be a purely respiratory one. The pair of slits in the mermaid's purse previously mentioned must serve for the conveyance of sea water into the interior of the purse where it probably mingles with the layer of albumen surrounding the developing embryo. Although it has been stated that these gill filaments absorb fluids which are milk-like secretions of the uterine mucosa and which serve as food for the growing embryo of all non-placental viviparous sharks and rays (Gudger, 1940) in the present species this is not one of their functions. Beard (1890, p. 310) states—"In the Skate-embryo the filaments are said to disappear shortly before hatching. It may be expected that their atrophy commences when the purse ruptures sufficiently to allow of the passage of sea water directly to the embryo. Then the ordinary piscine mode of respiration would be initiated, and the external gills would disappear." In the present species, however, they have already disappeared during the yolk sac stage when the embryo is 50 days old and the yolk sac has decreased to about half its original size (Plate IV, Fig. 9) and the internal gills are by this time well developed and functional.

At 104 days the embryo has absorbed all the yolk and the yolk sac has shrunk to such an extent that only a vestige of the umbilical cord remains extending through the umbilicus for a length of about 2 mm. (Fig. 10, u.). At the time of hatching the ventral abdominal surface of the embryo between the umbilicus and the cloaca is richly supplied with blood vessels which form a reticulation all over this surface (Fig. 10). The umbilicus persists for about 14 days after hatching and then completely disappears and the fish is fully formed (Fig. 11) and able to fend for itself, swimming about actively. The gestation period of the embryo which developed inside the egg case was also 104 days and the state of development at the time of hatching was the same.

SUMMARY

1. *Haploblepharus edwardsii* is an oviparous dogfish endemic to South African seas.

2. The male and female generative systems are dealt with and specialized features such as the albumen gland, nidamental gland, and the claspers are described.

3. Development takes place in an egg case or mermaid's purse, the gestation period lasting 104 days.

4. The development of an embryo external to the egg case is described.

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NARCOSIS AND CELL DIVISION IN COLPODA STEINII

RICHARD L. BURT[1]

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INTRODUCTION

For many years it has been known that a great number of chemical and physical agents can modify the course of mitosis and induce the development of abnormal division figures. Among the many experimental agents that have been employed to upset normal cell division are the following: basic dyestuffs (Politzer, 1924), narcotics (Politzer, 1931; Nemec, 1904; van Regemorter, 1926; Shaklevich, 1938; Ludford, 1936; Geiersbach, 1939), ether (Häcker, 1900; Schiller, 1909; Rosenfeld, 1932), alcohol (Krantz, 1938), X-radiation (Alberti and Politzer, 1923; Pfuhl and Küntz, 1939), radium (Whitman, 1933), ultraviolet radiation (Stevens, 1909), high and low temperatures (Bury, 1913; Bleier, 1930; Vintemberger, 1930; Kemp and Juul, 1931), and hypotonic solutions (Lewis, 1933). The instructive point to be gathered from a survey of such studies is that, irrespective of the agent employed, the induced morphological changes in cell division are quite similar. Thus, within certain limits of concentration or intensity of the experimental agency, disorientation of the chromosomes in the division figure, delay in polar movement and scattering of the chromosomes, "amitosis," pyknosis, dissolution of the achromatic figure, inhibition of cytokinesis, and formation of bi- and multinucleate cells are common effects.

The relative lack of specificity in results produced by such diverse experimental treatment makes the formulation of any complete explanation for these phenomena difficult. However, the suggestion of Ludford (1936) to the effect that metabolic changes may have a bearing on the production of anomalous divisional behavior becomes significant in light of recent observations indicating that special portions of the cell's metabolism are directly associated with certain physiological activity states (For details see Bodine (1934); Robbie, Boell and Bodine (1938); Deutsch and Raper (1938); Goddard and Smith (1938); Horowitz (1940); Pease (1941); Allen and Goddard (1938); van Schouwenberg (1938); Clowes and Krahl (1939); MacLeod (1941); Ormsbee (1941), and Fisher and Stern (1942)). From these studies it becomes apparent that a definite relationship exists between a portion of the over-all metabolism and cell or tissue activity. The use of narcotics as respiratory poisons and the deductions made by Fisher and his associates from data obtained by this type of treatment are of considerable significance in connection with this problem. The present study is an attempt to relate changes in cellular metabolism during division with the appearance of abnormal mitoses. The results of this study are in essential agreement with those of Fisher et al in that a respiratory parallelism can be demonstrated in *Colpoda steinii* by use of ethyl carbamate or chloral hydrate. The "activity system" of Fisher (that portion of the overall respiration that is most sensitive to narcotic inhibition) appears to be somehow associated with the maintenance of normal mitosis, for when it is differentially suppressed by appropriate narcotic concentrations, abnormal nuclear figures appear. These aberrant mitoses are believed comparable to those described in the literature that have been induced by many different agents. Barring such experimental intervention, the nuclear complex of *Colpoda steinii* is remarkably stable, unlike certain other members of the family Colpodidae (Burt, Kidder and Claff, 1941). Because of this stability it was selected for the study to be described.

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[1] Present Address: Harvard Medical School, Boston, Mass.

I am indebted to Professor George W. Kidder, Arnold Biological Laboratory, Brown University, for his stimulating interest during the course of this investigation.

Materials and Methods

Colpoda steinii was employed as experimental material throughout this investigation (for species designation see Burt, 1940). Although this ciliate cannot be readily grown in the absence of bacteria (food organisms) it offers several distinct advantages over other forms, notably, its rapid growth to high concentrations and the clarity of the mitotic changes during division (Burt, Kidder and Claff, 1941).

The ciliates were grown in sterile distilled water seeded with the coliform bacterium *Aerobacter cloacae.* One liter Erlenmeyer flasks were used as culture vessels. The procedure employed in culturing was as follows: 400 mls. of distilled water were placed in the flasks which were then plugged and autoclaved at 15 lbs. pressure for 20 minutes. Twenty-four hour Kolle flask cultures of *A. cloacae* on agar were used as food. The bacterial growth of two such Kolle flasks was harvested in 30 mls. of sterile distilled water, and 10 mls. of the resulting suspension were added aseptically by means of a pipette to each of two Erlenmeyer flasks containing the sterile distilled water. In this manner fairly uniform suspensions of food organisms were obtained. Stock cultures of *C. steinii* were carried in tubes with Aerobacter as food. Each flask was inoculated with one ml. of these tube cultures containing 12 hour cultures of ciliates (in the logarithmic growth phase). The Erlenmeyer culture flasks were then incubated 24–30 hours at room temperature before the ciliates were sacrificed. By adhering to this procedure an abundance of logarithmic actively dividing cells could be consistently obtained.

All culture vessels, pipettes and miscellaneous glassware employed in culturing the ciliates were autoclaved 20 minutes at 15 lbs. pressure to prevent extraneous bacterial contamination inasmuch as optimum growth of *Colpoda steinii* obtains with Aerobacter alone as the food organism (Kidder and Stuart, 1939). Throughout all cultural procedure rigid bacteriological technique was followed to obviate the difficulties arising from contamination of the cultures.

In the cytological studies the cells were removed from the culture flasks and centrifuged at slow speed for four minutes in 50 ml. centrifuge tubes. The supernatant was then removed by means of an aspirator and more organisms added to the tubes from the culture. Following a second centrifugation, giving adequate numbers of organisms, the ciliates were placed in 250 ml. Erlenmeyer flasks containing 50–75 ml. of freshly bacterized solution of narcotic whose effect was to be tested. Equal amounts of bacterial suspensions were added to each experimental and control solution in making up the dilution of the reagent employed. When the desired time of treatment had elapsed (one hour) the cells were again packed, the supernatant removed, and the cells placed on coverslips by means of capillary pipettes. It was found convenient and satisfactory to employ 25 per cent acetic acid in absolute alcohol for fixation. The nuclear details were found well preserved after fixation for five minutes in this reagent. The Feulgen technique was used exclusively in making the cytological preparations. This was necessitated by the fact that differentiation of the nuclear details is difficult with haematoxylin or other stains because of the retention of the dye by the division cyst walls. Best staining results were obtained by hydrolyzing in N/1 HCl at 60° C. for 12 minutes and staining in the fuchsin sulfurous acid for six hours. The stained preparations are then washed three times for four-five minutes each in HCl-Na-Bisulfite solution, placed for $\frac{1}{2}$ hour in running tap water, passed through the lower alcohols to 95 per cent and stained with fast green, dehydrated in absolute alcohol, cleared in xylol and mounted in damar.

It was customary to run these cytological experiments in four sections as follows: by centrifuging in four tubes the contents of the mass culture flasks were roughly quartered. The organisms obtained by centrifugation from three of these tubes were employed experimentally and accordingly treated with various concentrations of reagent. The cells from the fourth tube were always set up with bacterized distilled water and thus served as controls for the treated organisms. The control ciliates were always mechanically treated in a manner identical with that received by the experimental organisms. By this method aberrations in the mitotic or divisional processes could always be attributed safely to the effect of the inhibitor used.

For the respiration studies two 24–30 hour cultures set up as described were centrifuged and the cells so concentrated were resuspended in bacterized M/200 phosphate buffer at pH 7.0. One and one half mls. of this cell-buffer suspension was added to the Warburg vessels. Following equilibration (15 minutes) four readings were taken at ten minute intervals to determine the normal uninhibited respiration. The graded concentrations of inhibitor to be tested (made up in M/200 phosphate buffer at pH 7) were then dumped from the side bulbs and following a second equilibration period of ten minutes readings were again taken at ten minute intervals for one hour to determine the extent of respiratory inhibition. Temperature was controlled at 25° C. ± 0.1° C.

It was considered advisable to set up three vessels with M/200 phosphate buffer in the side bulbs to serve as controls for the experiments. In addition to these normal controls two more vessels were run with 1.5 ml. suspension of Aerobacter equivalent to the bacterial suspension employed in the experimental vessels. The final computed values for oxygen consumed per hour could therefore be corrected for not only an increase or decrease in normal control respiration but also for the negligible amount of oxygen consumed by the food organisms both before and after inhibition. The fact that organisms of the genus Colpoda encyst when the supply of food bacteria is depleted (Taylor and Strickland, 1938; Kidder and Stuart, 1939) necessitated the addition of bacteria to the experimental suspensions. Because of the presence of the bacteria, absolute values of oxygen consumed by the ciliates would be very difficult if not impossible to ascertain. However, where only relative rates of respiration are sought, as in this instance, the food organisms introduce no serious technical difficulty.

A simple technique was employed as a means of testing the degree of growth suppression in different concentrations of inhibitor. Although this method lacks the refinement of a cell counting technique, the results are believed to be roughly comparable. In these experiments four tubes were set up for every narcotic solution to be tested. The final volume of the narcotic solution following bacterization was 3 mls. In addition to this series of four tubes for every narcotic concentration tested through the range under investigation, four more tubes were set up with bacterized distilled water to serve as controls. Following inoculation with one loopful of logarithmic Colpoda, the growth in each of the four inhibited series and controls was read at 12, 24, and 36 hours. Control growth was arbitrarily designated as four plus, and growth in the experimental tubes designated as three plus, two plus, one plus, plus-minus, and minus depending on the degree of inhibition. The range of inhibitor concentration where growth was completely suppressed was termed the zone of complete growth inhibition. In these experiments aseptic procedure was not adhered to in either experimental or control tubes as over the period of time these determinations were made it was felt that extraneous bacteria would not significantly modify the results.

In assaying the effectiveness of the narcotic concentration tested in disrupting mitosis only cells in which the polar migration of daughter micronuclei was obviously retarded were counted as affected (compare Fig. 1-*H* with Fig. 1-*E*). As will presently be pointed out in detail, *Colpoda steinii* usually divides twice within a division cyst wall to produce four daughter ciliates. The aberrant nuclear divisions were accordingly expressed as per cent of all cells counted in mitosis between the first metaphase and the telophase of the second division. This method offered a reliable means of obtaining a quantitative estimate of the damage induced by the agents employed. It is to be emphasized, however, that the delay in polar migration of the daughter micronuclei was not the only observable defect resulting from narcosis. It merely served as a convenient method of evaluating the extent of mitotic derangement. The other changes concomitant with the polar defect will be considered elsewhere.

Potassium cyanide, potassium ferricyanide, sodium arsenite, iodoacetate, various carbamates and chloral hydrate were tested at varying concentrations for their effect on the division mechanism. Ethyl carbamate and chloral hydrate were most thoroughly studied in this respect and were also selected as inhibitors for the respiration studies.

OBSERVATIONS

Normal division

The organisms of the genus Colpoda normally reproduce within division cysts (for details see Kidder and Claff, 1938; Burt, Kidder and Claff, 1941). The trophic ciliates (Fig. 1-*A*) round up prior to division, dedifferentiate and secrete the cyst wall within which the ensuing fissions occur, *C. steinii* usually dividing twice to form four daughter cells. These stages are depicted in Figure 1, *A–G*. The first changes in the nuclear complex marking the onset of division are to be found in the macronucleus. This organelle loses its trophic ellipsoidal shape, becomes rounded, and the polar chromatin aggregates break up into irregular masses attaining the general configuration shown in Figure 1-*B*. Meanwhile the micronucleus proceeds through the prophasic changes first marked by swelling then condensation of chromatin to form striae from which the chromosomes are formed. These micronuclear transformations culminate in the metaphase configuration shown in Figure 1-*B* with the chromosomes oriented parallel to the long axis of the spindle. At this stage the macronuclear chromatin may be considered as divided into two portions, one consisting of the irregular masses centrally disposed; the other, being peripherally located and in optical section, seems to be plastered to the macronuclear membrane giving rise to a beaded appearance. The anaphase is depicted in Figure 1-*C*. At the termination of metaphase, the division figure appears to break into two parts followed by the rapid movement of the daughter halves to polar positions at opposite sides of the macronucleus (Fig. 1-*D* to *E*). Upon displacement of the daughter micronuclei by 180° the macronucleus elongates and constricts centrally. Cytokinesis soon follows at the completion of which the daughter micronuclei immediately pass into prophase and the same series of events is repeated with the result that four daughter cells are produced as shown in Figures 1-*F* and 1-*G*. Motor and oral organelles are differentiated in the four daughters and swimming movements are taken up within the cyst wall until finally the cyst membrane is ruptured and the ciliates escape into the surrounding medium. No visible nuclear extrusion has been observed during divisional phases in this species (Burt, Kidder and Claff, 1941).

Experimental modification of mitosis

Striking differences were observed in the effectiveness of the various inhibitors employed in disrupting the divisional mechanism. Ethyl carbamate was found to be quite active in this respect, however, and because of various other advantages offered by this compound, notably its high solubility and effectiveness over a relatively wide concentration range, it was most extensively studied. Potassium cyanide, potassium ferricyanide, sodium arsenite (neutralized solution), and iodoacetate were without effect up to toxic concentrations. Related carbamates produced changes similar to those induced by ethyl carbamate and the results of treatment with chloral hydrate are believed to be entirely comparable to those caused by inhibition with the urethanes. In the following account the cytological changes induced by these substances will be considered.

Ethyl carbamate: Striking changes in the course of mitosis were obtained in concentrations of ethyl urethane ranging from 0.5 to 2.0 per cent. The treatment did not appear to affect a particular stage of mitosis, however, but inhibition of any divisional stage in progress at the time of exposure seemed to obtain. A similar observation was made by Ludford (1936) for this narcotic. In view of

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FIGURE 1. *Colpoda steinii* × 1400. *A,* trophic organism; *B–G,* normal division phases; *H–L,* aberrant divisions produced by 1.3 per cent ethyl carbamate. *B,* Micronucleus in full metaphase of first division. *C,* Micronucleus in anaphase. *D,* micronucleus moving to poles. *E,* Micronucleus continuing polar movement; macronucleus elongating. *F,* Micronucleus at metaphase of second division. *G,* Second division completed. Nuclei of daughter cells returning to trophic condition. *H,* Failure of micronuclear migration; macronuclear chromatin aggregrates more diffuse; reappearance of Binnenkörper. *I,* Similar to *H*; arrested polar movement of micronuclei; macronuclear chromatin diffuse; Binnenkörper material reappearing. *J,* Segregation of both daughter micronuclei into one cell following first division. *K,* Arrestment of division at micronuclear metaphase. *L,* Both micronuclei at one pole of elongated macronucleus.

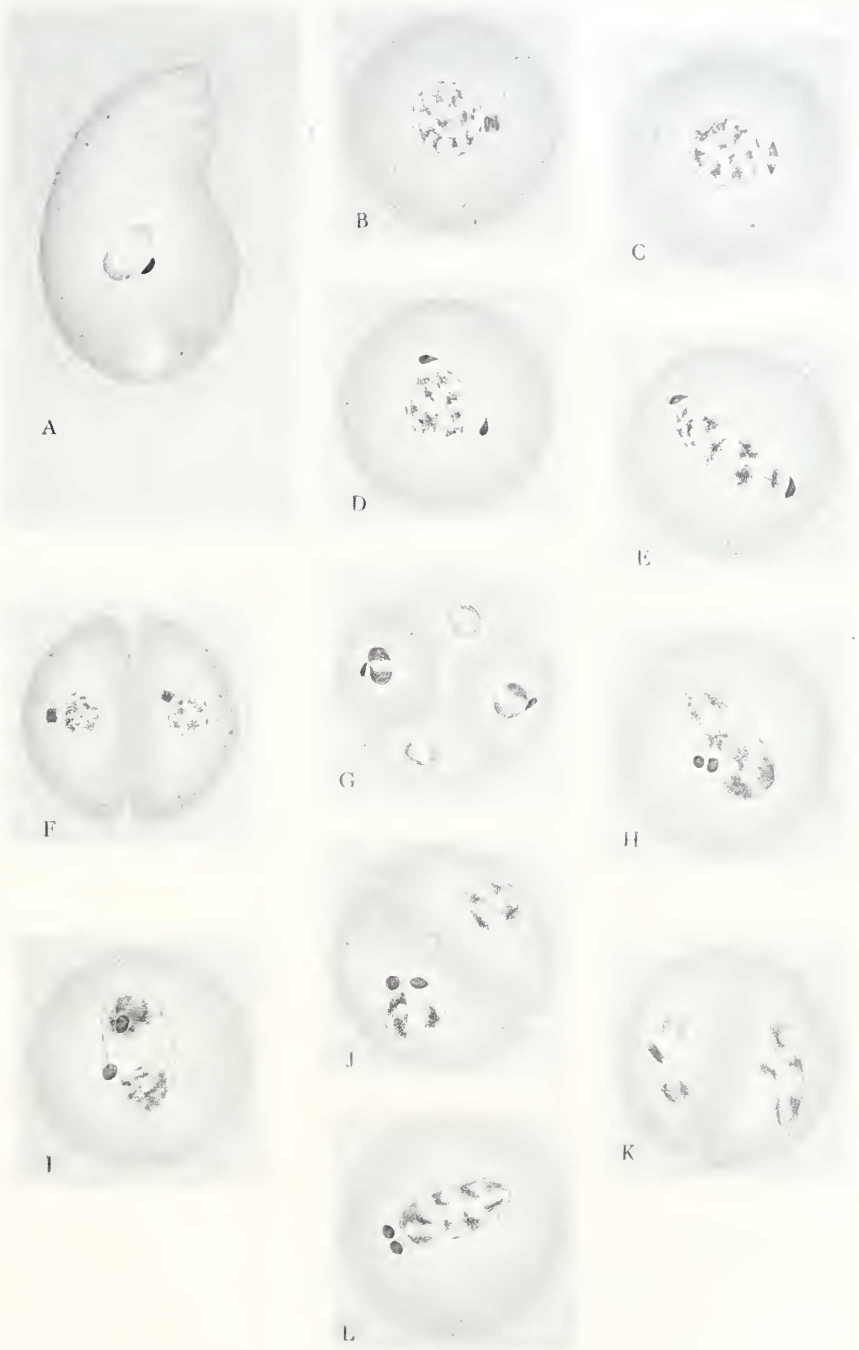
A
B
C
D
E
F
G
H
I
J
K
L

FIGURE 1

this fact, it is not at all surprising that a great variety of aberrant divisional types were observed. Because of this almost infinite variation in the nuclear features associated with narcosis, it is exceedingly difficult to evaluate the activity of the narcotic on any other basis than its effect on polar movement of daughter micronuclei.

With ethyl urethane this polar defect is exhibited through a concentration range of 0.5 to 2.0 per cent. Thousands of cells were counted following treatment with various narcotic concentrations (for complete data on this and the following observations see Burt, 1942). In Figure 2 these data are plotted semilogarithmically. The defective polar movement increases from 0 at 0.5 per cent urethane to a maximum of around 21 per cent at 1.5 per cent urethane concentration. This maximum value is probably determined by the number of ciliates in the first or second divisions whose micronuclei are between metaphase and telophase. During these stages one aspect of inhibition of cell division is expressed by lag in poleward movement.

As pointed out, however, the effect of urethane in appropriate concentrations on dividing *C. steinii* is characterized not only by delay of polar movement, but also by changes in the state of aggregation of both macro- and micronuclear chromatin and also by abnormal configurations of the dividing nuclear complexes. Some of these changes are illustrated in Figures 1-*H* to 1-*L*.

In Figure 1-*H* the polar defect is very well shown. At this phase of division, judging by the elongation of the macronucleus, the daughter micronuclei should have attained polar positions in the cell as is shown in Figure 1-*E*, a normal division figure. On the contrary, the daughters are centrally located near the middle of the macronucleus. A somewhat similar situation obtains in Figure 1-*I*, although the appearance is by no means as striking. In Figure 1-*L* another possible orientation of the nuclear complex is shown. Here the daughter micronuclei are terminally located at the same pole. One result of such aberrant behavior on the part of the daughter micronuclei is illustrated in Figure 1-*J*. In this instance cytokinesis and division of the macronucleus has occurred, but the micronuclei, due to failure in assuming polar positions, have both been segregated into one daughter cell. In Figure 1-*K* inhibition of division has occurred at the metaphase.

In all of these cases the aggregation of the macronuclear chromatin has been changed to some extent. In general, most of the macronuclear chromatin in the treated cells exhibits a tendency to become plastered to the nuclear membrane rather than maintain a central disposition characteristic of the normal dividing nucleus. Concurrently, the aggregates become more diffuse and varying degrees of fusion are shown (Fig. 1-*I*).

A noteworthy feature of the urethane-treated cells is the behavior of the Binnenkörper or plasmasomal material. Normally this substance, which stains lightly with fast green in tropic ciliates, is not in evidence in preparations immediately after the onset of nuclear division. However, in narcotized cells it reappears and is found dispersed around the diffuse chromatin aggregates of the macronucleus.

The morphological behavior of the chromatin and plasmasomal material in narcotized cells is suggestive of a partial reversion to a trophic nuclear condition. It is possible that under the influence of the narcotic and consequent suppression of normal nuclear division this tendency, although normally expressed at the termination of cell division, becomes dominant. At any rate, the appearance of the inhibited cells in no way suggests an *immediate* inhibition of all nuclear activity.

It is more likely that certain changes continue and give rise to the modified division figures observed.

Indicative of the fact that any phase of cell division is susceptible to the effects of narcosis is the diversity of aberrant division figures produced as a result of treatment. A continuous series of aberrant figures representing all stages of division may be constructed.

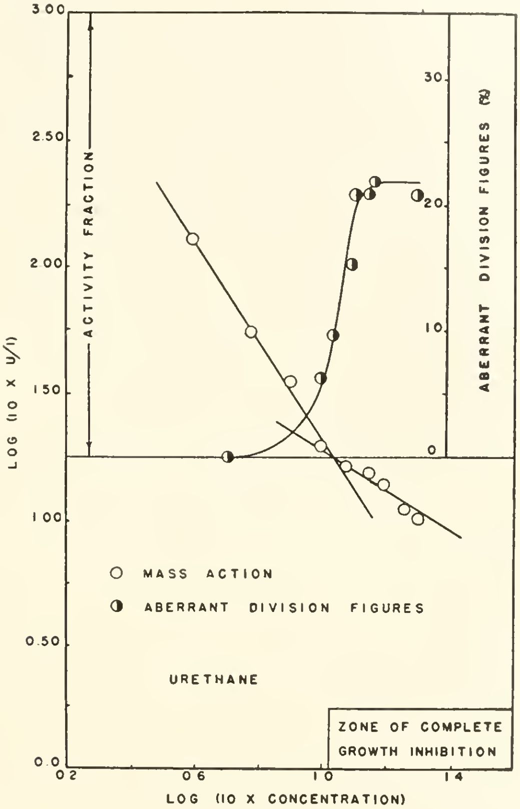

FIGURE 2. Summary of complete urethane data. Log ($10 \times U/I$), aberrant division figures (per cent) and zone of complete growth inhibition plotted against Log ($10 \times$ concentration of urethane).

Other carbamates: The affects of n-propyl, phenyl, n-amyl, iso-amyl, and ethyl-n-methyl carbamate on cell division were also studied. None of these substances were tested through as wide a range of concentration in small graded increments as was ethyl carbamate. However, it was found that these closely related compounds produced divisional changes qualitatively identical with those of ethyl urethane. The ability to inhibit micronuclear migration is common to all members of this group of substances. Similarly, the associated changes in arrangement and aggregation of macronuclear chromatin and the bizarre division figures described for ethyl urethane were also induced.

Chloral hydrate: The activity of this compound in disturbing the normal progress of division was assayed in graded concentrations of 0.1 per cent increments between 0.05 and 0.5 per cent. The morphological changes induced were similar to those described for the urethanes, but the percentage of chloralized cells in which the polar defect appeared was never high. Moreover, the concentration range for effective induction of the polar defect is quite narrow (0.1–0.3 per cent). The maximum number of deranged mitoses was only 3.9 per cent, a small value compared with the maximum ethyl urethane value of more than 20 per cent.

In excess of 0.3 per cent chloral hydrate the delay in poleward movement and the changes in the nuclei characteristic of cells narcotized in the lower concentrations rather abruptly disappear. In these higher concentrations retrogressive changes are induced which are expressed as condensation, diffuse staining and chromatolysis of the macronucleus and pyknosis of the micronucleus. The incidence of these changes, although slight to moderate at 0.4 per cent, increases rapidly with still higher concentrations until in 0.5–0.6 per cent chloral hydrate all of the organisms, both trophic and those in division, are frankly moribund. These degenerative changes are physiologically and morphologically reversible in some cells at least after one hour exposure at 0.6 per cent. If the cells are washed free of narcotic, packed by centrifugation and resuspended in freshly bacterized distilled water, growth will ensue. It is impossible to determine, however, whether or not every cell so treated is viable.

In summary, chloral hydrate affects cell division in a manner quite similar to ethyl carbamate and related compounds. However, the effective range of concentration for the production of these changes is relatively narrow, and at no concentration are the numbers of deranged divisions high. A possible explanation for this difference in activity will be outlined.

Effect of other inhibitors: The following compounds in the concentrations indicated were tested for their effect on the divisional mechanism:

Inhibitor	Concentrations
Potassium cyanide	0.005M to 0.000,05M
Potassium ferricyanide	0.050M to 0.005M
Sodium arsenite	0.001M to 0.000,25M
Monoiodoacetic acid	0.005M to 0.000,5M

These metabolic poisons throughout the concentrations designated were uniformly ineffective in the production of aberrant divisions of the type described for the carbamates and chloral hydrate. When cells were exposed to the lower concentrations of these inhibitors no change could be detected cytologically in the progress of division. However, at higher concentrations retrogressive changes characterized by extreme condensation and pyknosis of both macronuclei and micronuclei occurred. These changes were comparable to those induced at high concentrations of chloral hydrate and are considered as definite signs of cell damage as opposed to the milder type of change occurring with moderate narcotic concentrations.

Growth inhibition

As a correlative procedure in connection with the cytological and metabolic studies, the effect of ethyl urethane and chloral hydrate on suppression of growth in cultures of *C. steinii* was tested.

In Figure 3 are tabulated the results from one typical determination with ethyl urethane. Fairly close correspondence in the degree of inhibition among the four experimental series was obtained. It is apparent from these results that growth is only slightly affected by 0.5 per cent urethane (3 plus to 4 plus growth). However, with increasing concentrations of narcotic, suppression of growth becomes more pronounced until at 1.0 per cent urethane no cell division at all can be detected. Similarly, Ormsbee (1941) found that 1.0 per cent urethane would completely inhibit the growth of populations of the ciliate *Tetrahymena gelcii.*

CHLORAL HYD.		EXP. 1			EXP. 2			EXP. 3			EXP. 4		
SERIES	PERCENT CONC.	HOURS			HOURS			HOURS			HOURS		
		12	24	36	12	24	36	12	24	36	12	24	36
A	.025	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
B	.05	++++	+++	+++	+++	+++	++++	+++	+++	+++	+++	++	+++
C	.1	+	±	±	±	+	±	±	±	±	±	±	±
D	.2	–	–	–	–	–	–	–	–	–	–	–	–
E	.3	–	–	–	–	–	–	–	–	–	–	–	–
F	.4	–	–	–	–	–	–	–	–	–	–	–	–
G	CONTROL	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++

FIGURE 3. Inhibition of growth in cultures of *Colpoda steinii* with various concentrations of ethyl carbamate. Four plus growth represents growth in untreated controls.

URETHANE		EXP. 1			EXP. 2			EXP. 3			EXP. 4		
SERIES	PERCENT CONC.	HOURS			HOURS			HOURS			HOURS		
		12	24	36	12	24	36	12	24	36	12	24	36
A	0.5	+++	++++	++++	++++	++++	++++	+++	++++	++++	++++	++++	++++
B	0.8	+	+	+	+	++	++	+	++	++	+	+++	+++
C	0.9	±	+	+	±	±	+	±	±	+	+	+	+
D	1.0	±	±	±	±	±	±	±	±	±	±	±	±
E	1.1	–	–	–	–	–	–	–	–	–	–	–	–
F	1.2	–	–	–	–	–	–	–	–	–	–	–	–
G	CONTROL	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++

FIGURE 4. Inhibition of growth in cultures of *Colpoda steinii* with various concentrations of chloral hydrate. Four plus represents growth in untreated controls.

In Figure 4 are represented the results of an analogous determination made with chloral hydrate. Here complete inhibition of growth takes place in 0.2 per cent of the narcotic.

Comparison of the two sets of experimental results is instructive in indicating the differences in effective concentration ranges of the two inhibitors. In the case of ethyl carbamate an increment of 0.6 per cent in concentration must be made from the point where its effect is first noted until complete inhibition occurs (from 0.5 to 1.1 per cent). With chloral hydrate this increment is only 0.15 per cent or one fourth that of urethane. This is correlated with the brief range of effectiveness of chloral hydrate in the induction of aberrant division figures.

Respiratory metabolism

As pointed out in a preceding section, overall oxygen consumption in a cell or tissue does not necessarily have uniform significance as regards a specific function. Perhaps the most convincing evidence for this is the interpretation of nacrotic inhibition data made by Fisher and his colleagues (for review see Fisher, 1942).

Equations expressing the relationship between enzyme and inhibitor may be derived from the Law of Mass Action (Warburg, 1927; Warburg and Negelein, 1928; Fisher and Öhnell, 1940). As required by the Mass Action formulation, when the logarithm of the concentration of inhibitor (narcotic) is plotted against the logarithm of $\frac{\text{Uninhibited respiration}}{\text{Inhibited respiration}}$ a straight line should result. Nonlinearity of mass action curves so constructed is believed to indicate the presence of at least two parallel respiratory systems in Arbacia eggs and in yeast (Fisher, 1941a, b; Fisher and Henry, 1940; Fisher and Stern, 1942).

The respiration of *Colpoda steinii* was tested in graded concentrations of urethane and chloral hydrate with the object of obtaining evidence for such respiratory discontinuity in this organism. The results of respiratory inhibition as measured by the Warburg technique between concentrations of 0.4 and 2.0 per cent urethane are plotted in Figure 2. At a concentration of urethane between 1.0 and 1.2 per cent a break in the mass action curve occurs at which point respiration is inhibited by 35 per cent. This is taken as evidence for the existence of two separate respiratory mechanisms that differ in sensitivity to narcotic inhibiton.

In Figure 2 is also shown the relationship between this respiratory data, growth inhibition, and aberrant nuclear behavior at various levels of narcotic inhibition. The increase in pathological cell divisions is related to the degree of suppression of the upper limb of the mass action plot representing the inhibition of the more highly narcotic-sensitive fraction of metabolism. It is also interesting to note the correspondence between the concentration of urethane giving complete growth inhibition (1.1 per cent) and the concentration at which the break in the mass action curve occurs (approximately 1.1 per cent).

A discrepancy exists in these relations, however, for the maximum number of aberrant nuclear divisions is not obtained until a concentration of 1.4 to 1.5 per cent urethane is reached. This may be due to experimental error in part, but the theoretically more attractive possibility is that an overlapping in the inhibition of the two respiratory systems occurs. In accordance with this view the resting system would begin to be inhibited before the activity system was completely suppressed. This point will be dealt with more fully in a following section. Apparently, however, following complete inhibition of the more narcotic sensitive fraction (activity system of Fisher) not only is growth of the culture suppressed, but the incidence of abnormal divisional figures attains a maximum.

In Figure 5 is summarized the effect on respiration, growth and the derangement of cell division brought about by graded concentrations of chloral hydrate between 0.0125 and 0.6 per cent. These results resemble those obtained with ethyl urethane. Non-linearity of the mass action curve is characteristic. Also a fair correspondence exists between the concentration giving complete growth inhibition (0.2 per cent) and the concentration at which discontinuity of the respiratory inhibition becomes apparent (0.1 per cent). Unlike urethane, however, the percentage of aberrant nuclear divisions induced by chloral hydrate is never high, the maximum being in the vicinity of 4 per cent. This maximum is not obtained until the cells are exposed to 0.3 per cent chloral hydrate, or 0.2 per cent in excess of the concentration at which discontinuity in the respiratory data is expressed. Again, a possible explanation for this may be an overlapping in the inhibition of the activity and resting systems whereupon after a certain concentration is exceeded, both are inhibited simultaneously. That the more sensitive fraction indicated by chloral hydrate represents about 45 per cent of the total respiration (taking the break in the curve as the end point) in contrast to 35 per cent with urethane makes this possibility plausible. The higher value of respiratory inhibition down to the

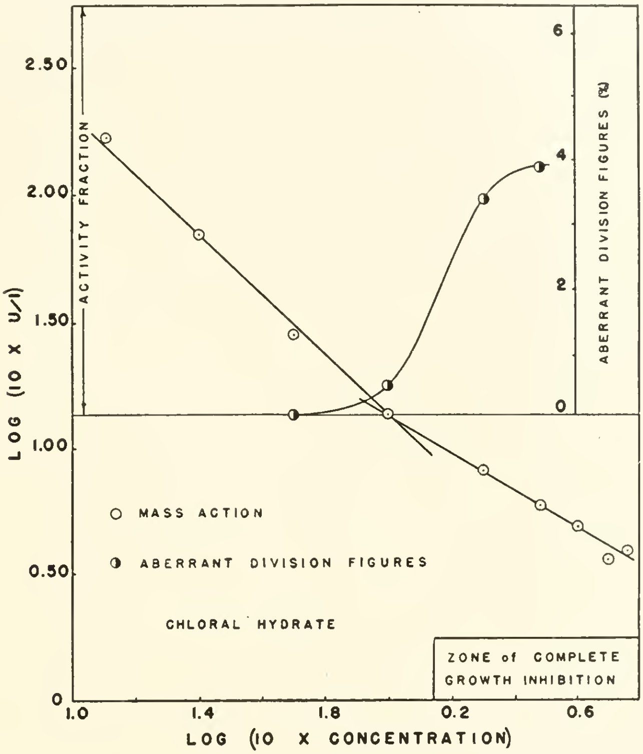

FIGURE 5. Summary of complete chloral hydrate data. Log $(10 \times U/I)$, aberrant division figures (per cent) and zone of complete growth inhibition plotted against Log $(10 \times$ concentration of chloral hydrate$)$.

point where discontinuity is displayed in the mass action curve therefore represents the activity system plus the added respiratory decrease caused by the suppression of part of the less sensitive fraction.

DISCUSSION

It is believed that under the experimental conditions adhered to in this study the data indicate the presence of at least two fractions of metabolism in *Colpoda steinii*. These differ in their sensitivity to inhibition by either chloral hydrate or urethane. One portion of this parallel respiratory mechanism, the activity fraction, appears to be associated with the maintenance of normal cell division. In general, this is in agreement with the observations of Fisher and his colleagues (1940–1942). This conception is based on the fact that discontinuity in the relation between concentration of narcotic and its effect in depressing respiration of these ciliates is expressed by mass action treatment of the data. The factors that might invalidate such an interpretation have been pointed out by Fisher and Stern (1942), but it is believed that these factors were not operative in this investigation.

Complete and quantitative separation of activity and resting fractions of the total metabolism by differential narcotic inhibition is considered here as extremely unlikely. Four findings in this report support the view that parallel inhibition of the two systems occurs in an intermediate range of narcotic concentrations:

1. The increase to a maximum in the incidence of nuclear aberrations after mass action discontinuity has been expressed. This is considered most significant and was found with both urethane and chloral hydrate (See Figs. 2 and 5).

2. The difference of 10 per cent between the activity respiration indicated by urethane (35 per cent) and that of chloral hydrate (45 per cent).

3. The delay in obtaining complete growth suppression with chloral hydrate after discontinuity in the mass action curve has been expressed.

4. The brief effective concentration range of chloral hydrate in inducing abnormal division figures and the low maximum for the polar defect obtained with this reagent.

Nuclear divisional activity persists at narcotic concentrations in excess of those at the break in the mass action curves. The maximum number of nuclear abnormalities with urethane does not occur until a concentration of 1.5 per cent of the narcotic is reached, and 0.3 per cent chloral hydrate is necessary to attain the corresponding maximum. Hence, the relation between the activity fraction, as determined by the respiratory inhibition curves, and divisional activity is not precise. It is believed that this failure of complete correspondence between the production of aberrant mitoses and discontinuity of the respiratory data is due to parallel or simultaneous inhibition of both activity and resting systems in an intermediate range of narcotic concentrations. In effect, this means that continued nuclear activity is possible even after the break in the mass action curves occurs because the activity system is not completely suppressed at this point. Mitosis therefore continues in an increasingly abnormal fashion until the activity metabolism is finally completely eliminated.

The conception of overlapping or parallel inhibition of both respiratory mechanisms also implies that the slopes of the two lines describing the reactions cannot be accurately descriptive of the relation between the narcotics on the separate systems even though the existence of at least two respiratory mechanisms is apparent. Furthermore, the value of 35 per cent representing the activity respiration, as determined by urethane inhibition, is not an absolute value for the percentage of the total energy involved in cell multiplication. However, this figure corresponds fairly well with the 28 per cent found for *Tetrahymena geleii* by Ormsbee (1941) and the 30–40 per cent of the overall respiration associated with the maintenance of cell division in sea urchin eggs reported by Krahl and Clowes (1939).

The value of 45 per cent for activity respiration, determined by chloral hydrate, can be accounted for by overlapping in the inhibition of the two respiratory mechanisms. The additional 10 per cent activity respiration indicated by this drug would thus represent part of the resting respiratory mechanism that was involved at the narcotic concentration where the break in the mass action curve occurred. Failure to completely suppress growth at a concentration of 0.1 per cent chloral hydrate (at break in logarithmic plot) likewise is indicative of this inhibition overlap. With the increase in narcotic to 0.2 per cent cell multiplication ceases because of the more complete elimination of the activity system.

When the progress of mitosis is suppressed by appropriate narcotic concentrations nuclear changes described earlier in this report as reorganizational are induced. Higher concentrations of narcotic inhibit these trophic changes (as 3 per cent urethane or 0.5 per cent chloral hydrate). At these concentrations the nuclei become condensed and pyknotic. The morphology described as resulting from varying degrees of suppression of the activity system obtained at lower narcotic concentrations are not seen at these higher drug levels. Thus in the production of nuclear abnormalities of the type described, two mechanisms appear to operate:

1. The inhibition of the activity system with associated suppression of the progress of mitosis.
2. Reorganization of the nuclei toward the trophic condition at that stage in division where inhibition has occurred.

The trophic changes are considered here to be dependent upon the maintenance of the resting metabolism of the cell (narcotic resistant system). As more of this less sensitive respiratory system is inhibited with increasing narcotic dosage, a point is finally reached when reorganization toward the trophic morphology cannot occur. Concomitantly pyknosis sets in and the nuclear picture is completely altered. Where the resting fraction is inhibited simultaneously with the activity fraction a restriction on the effective production of aberrant divisional figures results. The brief range of effectiveness and low maximum for nuclear abnormalities obtained with chloral hydrate can be accounted for on this basis.

The conception of overlapping with respect to narcotic inhibition postulated in the foregoing account is supported by the observation of Fisher (1941a) that benzoate inhibits the resting system in sea urchin eggs before inhibiting the activity system. Here it is necessary to inhibit 50 per cent of the respiration before inhibition of cleavage occurs. The condition described for *C. steinii* in its behavior with narcotics appears to represent an intermediate situation between the theoretical, where precise quantitative separation of the two systems would occur, and the juxtaposition of the two systems with respect to sensitivity to benzoate described for sea urchin eggs.

It is to be emphasized that the narcotic concentration required to inhibit cell multiplication in cultures corresponds closely with that at the point in the mass action curves where discontinuity is expressed, i.e., when the activity system, as determined by this method, is inhibited growth of the culture ceases. To suppress nuclear activity, however, more complete elimination of the activity system appears to be necessary, hence the delay in attaining the maximum number of abnormal division figures.

The cytological effects of narcotics on dividing *Colpoda steinii* are considered here as unique, but are comparable to the results obtained with these agents on many other cell types. Furthermore, it is apparent that in addition to narcotics many chemical and physical agents induce quite similar changes in the course of cell division. In general, the interpretation placed on this non-specificity of effect is that regardless of the agent employed there is somehow brought about a modification of the intracellular colloid state. This common result of experimental treatment would account for the similarities of morphological variation (see Ludford, 1936; von Lehotzky, 1938; Kemp and Juul, 1931; M. R. Lewis, 1933, 1934; Rosenfeld, 1932). This point of view, in light of the paucity of information available that is pertinent to the actual mechanics of these defects, appears to be well taken. However, the complexity of the protoplasmic organization in its reactions to various treatments must not be overlooked.

In the present study it has been shown that with gradual suppression of that portion of the cells' energy yielding reactions designated as activity metabolism, cell multiplication is suppressed and abnormal division figures are induced. It is not surprising that the divisional mechanisms drawing on these energy sources should reflect such reductions in energy production by abnormal behavior. However, it is difficult to visualize the manner in which this metabolic inhibition becomes morphologically expressed.

Possibly by interfering with cellular oxidation (narcosis) physical changes in the protoplasm result (von Lehotzky, 1938); this would relate the abnormal division figures obtained by narcotics to those induced by temperature changes, ether, carbon dioxide, hypo- and hypertonic solutions. All of these agencies have been demonstrated to affect the viscosity of protoplasm (for review, see Chamber's discussion in Cowdry's *General Cytology*). Conceivably, such experimental interference might exert its effect by modifying normal cyclical viscosity changes occurring during cell division (Heilbrunn, 1917; 1920; 1921).

No doubt such physical changes are significant for the normal progress of cell division and bear consideration in dealing with experimentally induced changes in mitosis. That such purely physical aspects fail to describe the entire sequence of events encountered in the complex dynamics of cell division is obvious. It may be, however, that they are the links between the biochemical phases involving cellular oxidations and the well known cytological manifestations of activity. Integrating factors for such an association are at present lacking. With regard to the present study, it can only be said that a certain portion of the overall metabolism is probably associated with these mechanisms. To postulate more than this would be the sheerest speculation.

Briefly, the negative results obtained in the preliminary work with cyanide, ferricyanide, arsenite and iodoacetate may be considered. The reactions of any of these inhibitors in the intact cell, with the possible exception of cyanide, are complex. At the present time data concerning their effect on the metabolism of *Colpoda steinii* are lacking. Apparently, however, with the limited information concerning the morphological expression of treatment by these agents, their effects differ considerably from those of the narcotics tested. To take one example, cyanide does not seem to visibly alter the division mechanism until the occurrence of retrogressive changes resembling those induced by the higher doses of narcotics. Tentatively it may be suggested that cyanide, by virtue of its reaction at the oxygen end of the cellular oxidation chain where a large percentage of respiration is mediated, simultaneously inhibits both activity and resting systems. When a certain percentage of the resting metabolism has been suppressed, the retrogressive alterations are induced.

Summary

1. Ethyl carbamate has been demonstrated to be effective in inducing aberrant nuclear behavior during division of the ciliate *Colpoda steinii*. This activity is shared by other carbamates and chloral hydrate. These effects are described in detail.

2. A quantitative means of assaying cytologically the activity of these substances is described. On the basis of defective polar movement of daughter micronuclei ethyl carbamate was found to be more active than chloral hydrate. A possible explanation for this observation is postulated.

3. The results of preliminary work involving treatment of dividing cells with potassium cyanide, potassium ferricyanide, arsenite, and iodoacetate are reported. These agents apparently do not share the activity evidenced by the carbamates or chloral hydrate.

4. By means of the Warburg technique the effect of urethane and chloral hydrate on respiration of *C. stenii* was studied.

5. Discontinuity in the quantitative action of these agents on respiration is interpreted to indicate the existence of at least two fractions of metabolism. One of these, the activity system, is believed to be associated with the process of cell division.

6. Complete separation of these two systems is not considered probable, however, and caution is urged in the interpretation of discontinuity in Mass Action treatment of this and similar data.

7. The significance of the association of the appearance of aberrant division figures, growth inhibition and discontinuity in the relationship of narcotic concentration to respiratory inhibition is pointed out. Discrepancies in these relationships are discussed.

8. The bearing of metabolic inhibition on the general problem of the pathology of mitosis is discussed.

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A NOTE ON THE ABSORPTION SPECTRA OF THE BLOOD OF EUDISTYLIA GIGANTEA AND OF THE PIGMENT IN THE RED CORPUSCLES OF CUCUMARIA MINIATA AND MOLPADIA INTERMEDIA

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In 1868 Lankester noted the presence of a red pigment (erythrocruorin) in the plasma of certain annelids, in Chironomus larvae and in Planorbis. He also reported a green pigment (chlorocruorin) in the plasma of Siphonostoma and Sabella. Subsequent studies have revealed the widespread occurrence of erythrocruorin[2] (invertebrate hemoglobin) in many worms, in echinoderms (holothuroids), in some molluscs and in a few arthropods (Redfield, 1933; Kobayashi, 1936).[3] Chlorocruorin appears to be more limited in its distribution having been found thus far only in certain polychaete worms (Fox, 1925).

The absorption curves of both these heme pigments have been studied in some detail by Fox (1925), Redfield and Florkin (1931) and Kobayashi (1932, 1935, 1936). From a review of this literature it appears that the α-band of oxyerythrocruorin falls, in most cases, well within the 577–579 mμ region, although figures as low as 574.5 mμ have been reported for some of the worms (Barcroft and Barcroft, 1924; Kobayashi, 1936). The usual position of the α-band agrees well with the position of the comparable band in vertebrate blood. The β-band of oxyerythrocruorin occurs, in most cases, between 540–542 mμ although, here again, there are exceptions as in the case of the holothuroid, *Caudina chilensis,* where this band is found at 544.2 mμ (Kobayashi, 1932) and in the case of the earthworms, *Pheretima communissima* and *Pheretima hilgendorfi,* where the band occurs at 538 mμ and 539 mμ respectively (Kobayashi, 1936). The usual position of the β-band of oxyerythrocruorin agrees generally with the position of the comparable band in vertebrate blood. Reduced erythrocruorin, in many cases, possesses a single band with maximum at 556 mμ, which is also the case for vertebrate hemoglobin, but in *Caudina chilensis* this maximum has been reported to occur at 560 mμ (Kobayashi, 1932) and in *Cucumaria frauenfeldi* at 558 mμ (Hogben and Van Der Lingen, 1938). The situation appears to be distinctly different for some of the worms where the reduced pigment possesses a double peak; one band being at 566–571 mμ, the other at 549–551 mμ (Kobayashi, 1936; Vlès, cited by Kobayashi, 1936).

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[2] Many authors, since the original work of Lankester, have used the term hemoglobin for the pigment in invertebrates, but the present writer is following the suggestion of Svedberg and Eriksson (1933) that, since the protein portion of invertebrate hemoglobin is characteristically different from that of vertebrate hemoglobin, the separate name, erythrocruorin, is justified.

[3] A report has been published by Sato and Tamiya (1937) indicating bands of hemoglobin in *Paramecium caudatum,* but the writer has seen only an abstract of this report.

Compared to oxyerythrocruorin, the bands of oxychlorocruorin are shifted toward the red end of the spectrum. In five species of worms, Fox (1925) obtained a spectrum with the α-band in the 602.5–605.9 mμ region, while the β-band (in Spirographis) is at 561 mμ and a third faint band (also in Spirographis) is located at about 517 mμ. Using crystalline chlorocruorin, Roche and Fox (1933) have been able to confirm these results.

Recently the author had occasion to examine spectroscopically the green pigment dissolved in the plasma of the tube worm, *Eudistylia gigantea,* and the red pigment of the corpuscles found in the perivisceral fluid of the holothuroids, *Cucumaria miniata* and *Molpadia intermedia.* The results which were obtained agree in many details with previous data, but in some respects the results are so strikingly different that a record of them would be of value, even though the war has prevented completion of the investigation.

The author wishes to express his appreciation to Professor Thomas G. Thompson for making available the facilities at the laboratories in Friday Harbor and the Oceanographic Laboratory in Seattle. To Professor Trevor Kincaid appreciation is expressed for his aid in identifying the animals used in this work.

Materials and Methods

The large tube worm *Eudistylia gigantea* (family Sabellidae) is found at relatively low tidal levels in large colonies attached to rocks or pilings in certain areas of Puget Sound. The pigment, which is dissolved in the plasma, appears red in the concentration occurring in the dorsal blood vessel, but upon removal and dilution it is seen to be green. About 0.1–0.2 ml. of blood was obtained by means of a glass capillary tube inserted into the dorsal blood vessel. After dilution to about 50–100 ml. with either cold distilled water or with cold sea water, the solution was filtered and the filtrate, containing the pigment, was placed in a 10 cm. long specimen tube. The spectrum was then obtained by visual matching, using a Bausch and Lomb spectrophotometer. The holothuroids which were used include the sea cucumber, *Cucumaria miniata* (order Dendrochirota), which is found abundantly at Friday Harbor in between rocks at low tide level, and the apodous sea cucumber, *Molpadia intermedia* (order Molpadiida), which was obtained from the muddy bottom of East Sound at a depth of 12–15 fathoms. The red pigment is located in numerous corpuscles suspended in the perivisceral fluid of both these sea cucumbers. These corpuscles in Molpadia, when examined microscopically, are seen to be pale-yellow cells which possess a variety of elongated, multi-lobed shapes. A few are oval or spherical in form and all of them have a single, small, dark and spherical nucleus located at a variety of points in the cell but rarely at the geometric center. When a drop of distilled water is mixed with a drop of the perivisceral fluid the cells assume a perfectly spherical form and the nucleus is seen to occupy an eccentric position. The corpuscles of Cucumaria are also pale-yellow, nucleated cells with a variety of shapes; some are ovoid, some spherical and others are quite irregular with one or several processes. The elongated lobed forms of Molpadia are seldom seen in *Cucumaria miniata.* Previous accounts of the holothurian red cell by Dawson (1933) and Ohuye (1936) have already mentioned some of these structural features. By means of a small puncture in the body wall, the red perivisceral fluid was collected and the cells centrifuged out. After decanting the supernatant fluid, the cells were washed in cold sea water and again centrifuged out. This process was repeated three or four times after which the cells were hemolyzed in cold distilled water. The solution of pigment after filtration and dilution to about one hundred ml. with cold distilled water was examined spectroscopically.

Results and discussion

The five curves (Fig. 1) obtained from five different sea cucumbers (*Cucumaria miniata*) give an indication of the reproducibility of the spectral curve and show that the location of a point of maximum or minimum can be checked within 2 mμ. These curves, which were obtained from well-aerated solutions of the pigment, show two maxima; one 580–581 mμ, the other at 544–545 mμ. The point of minimum absorption between these two maxima is at 564–565 mμ. A comparison of the spectral curves of the pigments, in the oxidized state, from all three species is shown in Figure 2. It is evident that all three pigments show two maxima, but the maxima for Eudistylia and for Molpadia are shifted toward the red end of the spectrum when compared with the Cucumaria data, the shift being greater in the case of Molpadia. The position of the maximum and minimum points for all the curves that were obtained from all three organisms are listed in Table I.

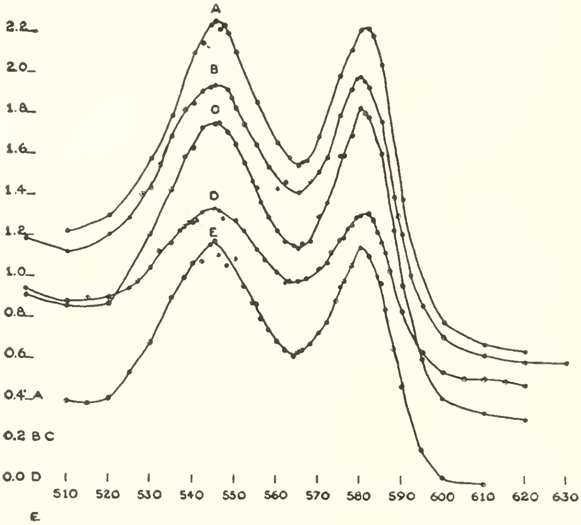

FIGURE 1. The absorption spectral curves of the pigment from five different sea cucumbers (*Cucumaria miniata*). The density (log I_0/I) is plotted as ordinates against the wave length, in mμ. The individual graphs are plotted on a multiple ordinate scale to avoid crowding. The zero ordinate for each curve is indicated by the corresponding letter. Each plotted point represents the mean of 3–5 spectrometric readings.

Reduction of the pigment by means of sodium hyposulphite results in a radical, though reversible, change in the spectral curve for all three pigments (Fig. 3). In the case of Cucumaria the original bands disappear and two new bands, one at 562–563 mμ, the other at 530–532 mμ, make their appearance. The point of minimum between these two maxima is located at 545 mμ (Table I). In Eudistylia, reduction results in the disappearance of the two original bands and in the appearance of a band with a peak at 577–580 mμ. There is also an indication of a secondary band at about 540 mμ (Fig. 3) but the data on this point are too meagre to merit any degree of confidence. Reduction of the pigment from Molpadia, again with hyposulphite, leads to the replacement of the two original bands by a single band at 588 mμ. It is possible that, here too, more data would reveal the existence of a secondary band since an indication of this is visible in the curve for Molpadia

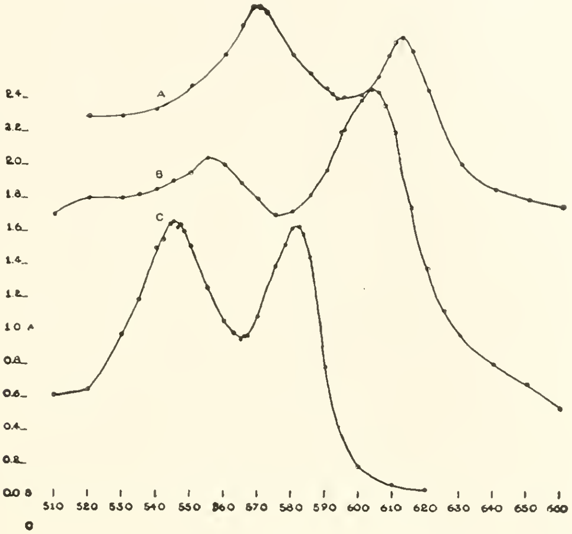

FIGURE 2. The absorption curves of the oxidized pigment from *Molpadia intermedia* (*A*), *Eudistylia gigantea* (*B*), and *Cucumaria miniata* (*C*). The curves are plotted on a multiple ordinate scale. Other details as Figure 1.

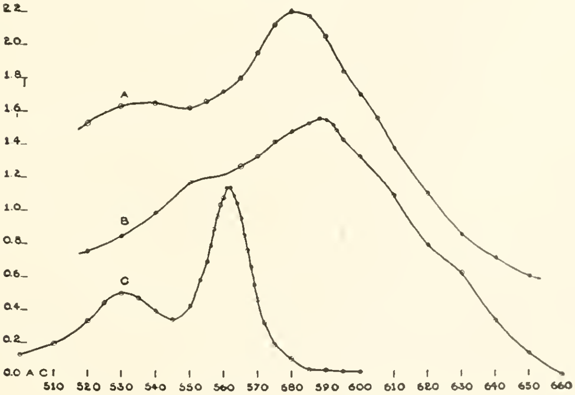

FIGURE 3. The absorption curves of the pigment after reduction with sodium hyposulphite from *Eudistylia gigantea* (*A*), *Molpadia intermedia* (*B*), and *Cucumeria miniata* (C). The curves are plotted on a multiple ordinate scale. All other details as in Figure 1.

Table I

Species *		Oxidized state			Reduced state	
		α	β	Minimum *	Main	Secondary
Caudina chilensis		579.5	544.2	564.5	560	
Molpadia roretzii		577	541.5	562	557	
Cucumaria frauenfeldi		579	543		558	
Spirographis spallanzi (crystals)		602.5	562.5			
Eudistylia gigantea	1	605	555	576	580	540
	2	604	555	576	577	
	3	603	558	577		
	4	602	554	575		
Cucumaria miniata	1	581	545	564	562	
	2	580	545	564	561.5	530
	3	580	544	564	562	531
	4	580	544	565	562	531
	5	580	547	565	562	531
	6	582	545	565	563	532
	7				563	535
Molpadia intermedia	1	615	570	592	588	
	2	612	570	592	588	
	3	611	568	595		

* The position of the minimum between the α- and β-bands.
The data from Caudina and *Molpadia roretzii* were taken from Kobayashi (1932).
The data for *Cucumaria frauenfeldi* were taken from Hogben and Van Der Lingen (1928).
The data for Spirographis were taken from Roche and Fox (1933).

(Fig. 3). It is clear, then, that reduction of the pigment from all three organisms leads to a hypsochromic shift, but the shift is not one of equal magnitude for all three organisms, since the span from the α-band to the principal band of the reduced pigment is about 18 mμ for Cucumaria, 25 mμ for Molpadia and 26 mμ for Eudistylia.

It seems very likely that the pigment in the red cells of *Cucumaria miniata* is erythrocruorin. The positions of the α- and β-bands, as well as the point of minimum, agree reasonably well with the previous data obtained with other holothurians (Table I). The reduced pigment from Cucumaria shows two bands; the main band at 562–563 mμ agrees approximately with the 557–560 mμ band previously reported for holothurians (Table I), but the secondary band at 530–532 mμ is previously unreported, although from the plasma pigment of certain worms (Kobayashi, 1936) a spectral curve has been obtained which has a 2-banded structure with the secondary band in the 549–551 mμ region.

It also seems likely that the green pigment dissolved in the plasma of *Eudistylia gigantea* is chlorocruorin. The α-band at 602–605 mμ (Table I) agrees completely with the data given by Fox (1925) and by Roche and Fox (1933), although the present data shows the β-band to be shifted toward the violet end of the spectrum by about 6–7 mμ as compared with the position of the β-band given by the above-mentioned workers. The principal band of the reduced chlorocruorin from Eudistylia has a peak at 577–580 mμ whereas the data of Fox (1925) for Spirographis place it at about 574 mμ.

The results obtained from *Molpadia intermedia* are surprising. At the outset there was no reason to suspect that an absorption curve agreeing approximately with that obtained by Kobayashi (1932) from *Molpadia roretzii,* and indicating an erythrocruorin, should not be obtained. Instead, as Figures 2 and 3 indicate, the bands for both the oxidized and reduced pigment are shifted toward the red end to a degree even greater than in the case of chlorocruorin. The α-band of *Molpadia intermedia* occurs 36 mμ further toward the red than the corresponding band for *Molpadia roretzii,* while the band of the reduced pigment of *Molpadia intermedia* is 31 mμ further toward the red than in *Molpadia roretzii.* It is also significant to note that the span between the α- and β-bands in other sea cucumbers (Table I) is 35–36 mμ whereas the corresponding span in *Molpadia intermedia* is about 43 mμ. These differences appear to be too great to be accountable to errors in measurement or to the usual species differences that are known to occur. The spectrum of the pigment from *Molpadia intermedia* does not agree with the spectrum of hemerythrin, the red pigment found in certain Gephyrean worms as well as in the polychaete, Magelona (Marrian, 1927). It must be concluded that either this represents the true absorption spectrum of a heme pigment (if it is a heme pigment) characteristically different from either erythrocruorin or chlorocruorin, or that unrecognized conditions cause a shift of the bands from the typical positions of erythrocruorin. The onset of war resulted in the sudden interruption of the investigation at this point so that this final question must remain unanswered till a later date. A thorough examination of the crystallized pigment by chemical as well as by spectroscopic means should be made before the existence of a new pigment can be accepted.

SUMMARY

A spectrometric examination of the green pigment dissolved in the plasma of the tube worm, *Eudistylia gigantea,* and of the red pigment in the corpuscles of the sea cucumbers, *Cucumaria miniata* and *Molpadia intermedia,* has led to the following conclusions.

1. The green pigment of Eudistylia appears to be chlorocruorin. In the oxidized state it possesses an α-band at 602–605 mμ and a β-band at 554–555 mμ. In the reduced condition a main band occurs at 577–580 mμ with a second band suggested at 540 mμ.

2. The red pigment of Cucumaria appears to be erythrocruorin. In the oxidized state an α-band at 580–581 mμ and a β-band at 544–545 mμ are seen. When the pigment is reduced a band appears at 562–563 mμ as well as one at 530–532 mμ.

3. The red pigment of Molpadia, when oxidized, possesses a band at 611–615 mμ and another at 568–570 mμ. In the reduced condition a band at 588 mμ is evident. This spectrum does not agree with the spectrum of either chlorocruorin or of erythrocruorin. The new spectrum may indicate the existence of another pigment with the ability to combine reversibly with oxygen.

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GUSTATORY REJECTION THRESHOLDS FOR THE LARVAE OF THE CECROPIA MOTH, SAMIA CECROPIA (LINN.)

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INTRODUCTION

Dethier (1937, 1939) and Eger (1937) have so far conducted the only studies on gustation in lepidopterous larvae. Dethier, interested in localizing the gustatory receptors and in gustatory acuity and its relation to host selection by caterpillars, has determined acceptance thresholds for certain sugars and rejection thresholds for hydrochloric acid for a number of species. Eger, interested chiefly in determining the modalities of taste for caterpillars, has determined acceptance thresholds for some sugars and rejection thresholds for hydrochloric acid, sodium chloride, acetic acid, quinine hydrochloride, and saccharine. He concludes that there are probably only two modalities for caterpillars—acceptable and unacceptable, but he admits that his data offer no definite proof for this. The following experiments were undertaken to add more information to that already existing in this little known field.

MATERIALS AND METHODS

The larvae of *Samia cecropia* used in the following experiments were reared from a single group of eggs laid by a gravid female captured in the early summer, the food plant being the sycamore (*Platanus occidentalis*). They were at first confined in a large jar and fresh leaves were added as needed, but later they were put on individual branches and numbered by marking with drops of colored paint. All the animals used in the experiments described below were in the last instar.

Rejection thresholds were determined, without disturbing the larvae, by placing drops of test solutions on the leaves as the animals fed. Since the caterpillars eat leaves in a very orderly fashion, it is easy to place drops so that they are reached by them within a very short time, usually about 10 to 15 seconds. Drops were placed on the leaves from small, new and carefully washed artists' brushes kept in the test solutions. A definite attempt was made to have the drops as nearly the same size as possible, but the importance of this is doubtful, for the larvae ordinarily ate on, undisturbed, below their thresholds and reacted almost immediately upon contact when the solutions were concentrated enough to bring about rejection.

The test solutions were prepared from C.P. chemicals, unless otherwise noted, using volumetric apparatus, and all tests were run within three hours after the solutions were prepared. The following chemicals were used, the concentrations prepared being given in parentheses after each: sucrose (2M, 1M, 0.5M), glucose, U.S.P. (2M, 1M, 0.5M), lactose, U.S.P. (1M, 0.5M), strychnine sulfate, U.S.P. (saturated solution), hydrochloric acid (0.4N, 0.2N, 0.1N, 0.075N, 0.050N, 0.025N), acetic acid (0.8N, 0.4N, 0.2N, 0.1N, 0.075N, 0.050N), sodium hydroxide

(0.4N, 0.2N, 0.1N, 0.075N, 0.050N), sodium chloride (2N, 1N, 0.75N, 0.50N, 0.25N), lithium chloride (4N, 2N, 1N, 0.75N, 0.50N), calcium chloride (1N, 0.75N, 0.50N, 0.25N, 0.10N), ammonium chloride (1N, 0.75N, 0.50N, 0.25N, 0.10N), and potassium chloride (1N, 0.75N, 0.50N, 0.25N, 0.10N).

There was no particular order in which these solutions were presented to the individual larvae. Since tests could be carried on only during feeding, it was found convenient to use three or four solutions simultaneously with all the caterpillars, testing the animals, whenever they fed, with the various concentrations. Thus there was no chance for associations to be built up by the caterpillars through consistent use of ascending or descending orders of concentrations. Each of the thresholds was determined at least three times for each of the caterpillars, and, in most cases, was determined five or six times, the greatest number for any individual being nine times. Acceptances are easy to determine, because the animals eat three or four "cuts" through the drop in their feeding. Rejections are usually equally sharp, because the caterpillars stop feeding immediately upon touching the drop, withdraw and start feeding elsewhere. It was possible, therefore, although only twelve specimens were used, to make accurate determinations for these solutions under these conditions.

The temperature of the laboratory was 23° C. and the relative humidity 70 per cent throughout all the work. Ordinary daylight entering the windows was the source of illumination.

RESULTS

There were no rejection thresholds for sugars or for strychnine sulfate, the larvae eating the most concentrated solutions of these offered to them. The thresholds for acids and salts used in these experiments are given in Table 1.

TABLE I

Substance	Range	Mean	$\frac{1}{\text{Threshold}}$
HCl	0.04–0.15 N	0.083 ±.008 N	12
$\mathrm{CH_3COOH}$	0.09–0.6 N	0.46 ±.05 N	
NaOH	0.09–0.15 N	0.13 ±.008 N	7.7
KCl	0.2 –0.9 N	0.45 ±.05 N	2.2
$\mathrm{NH_4Cl}$	0.4 –0.6 N	0.46 ±.03 N	2.2
NaCl	0.4 –1.5 N	0.89 ±.1 N	1.1
$\mathrm{CaCl_2}$	0.2 –0.9 N	0.61 ±.05 N	1.6
LiCl	0.9 –3.0 N	1.4 ±.15 N	0.72

In the column headed, "Range," are the lowest and highest thresholds discovered for the group; this does not imply a uniform distribution between these two extremes, but it gives some indication of the individual variations. In the column headed, "Mean," are the mean threshold normalities and the standard errors of these means (probable errors would be .6745 times the standard errors). In the column headed, "1/Threshold," are the reciprocals of the thresholds, these figures to be used below as indicative of stimulative efficiencies.

Thresholds were calculated as follows. The two critical concentrations—that is, the pair of concentrations, the lower of which was accepted, the higher rejected—were determined for each larva. Obviously, the true threshold lies between these two, and, for statistical purposes, it was assumed that the threshold lay midway between them. Thus, if a larva accepted 0.25N and rejected 0.50N, the threshold assigned was 0.38N, if between 0.50N and 0.75N, the threshold assigned was 0.63N. Since the second digit cannot be interpreted literally in such a system, these thresholds are designated in the table as 0.4N and 0.6N respectively, only the means being given to the second place.

While the validity of using the midpoint betweeen the two critical concentrations as the threshold for an individual animal can be questioned, certain supplementary observations and considerations made it appear permissible to use this for the calculation of means. Thus, most of the animals readily ate through the drop of lower concentration and stopped clearly on reaching the drop of higher concentration, indicating a threshold in the middle range between the two. A few, however, hesitated at the lower concentration before continuing with feeding and reacted violently at the higher, drawing back sharply and often ceasing feeding for a time, indicating a threshold near the lower concentration. Others ate readily through the lower concentration and only hesitated at first at the higher, made a few tries at eating it, but finally stopped and usually simply moved a short distance away on the same leaf, indicating a threshold near the higher concentration. Since these latter two cases seemed to be of equal occurrence, with the majority of rejections of the first type described, it seemed fair, for purposes of calculation of means for the group to be used for comparative purposes only, to assign the midpoints between the critical concentrations as the thresholds for the individuals, in the group, without implying that these represent true thresholds for any of the individuals.

Discussion

Sugars and strychnine sulfate

Since there were no rejection thresholds for sugars or for strychnine sulfate, no conclusions can be drawn concerning the ability of the caterpillars to taste these substances. It must be recognized that failure of rejection does not imply that the animals cannot taste these compounds, just as rejection thresholds do not measure the lowest concentrations that can be tasted. To me, sycamore leaves tasted almost as bitter as the strychnine solution, and, since these larvae feed on many species of trees, it would seem logical that their rejection threshold for bitter substances would be high. Dethier (1937) has shown definitely that caterpillars of this species can taste glucose and sucrose, but he used acceptance thresholds in his work.

Acids

The acid thresholds here given are higher than those given by Dethier and Eger, but that is to be expected, for the acids in their experiments were offered in drops of water, while in this case there was some mixing of the acids with the food. The pH of the solution of hydrochloric acid corresponding to the mean rejection threshold normality is 1.2, while the pH of the solution of acetic acid at the mean rejection threshold normality is 2.5. Thus, acetic acid, as for man, is much more effective a stimulating agent than is hydrochloric acid at the same pH. The ratio for this species is 20:1, which compares favorably with Eger's (1937) report for another species of a ratio averaging about 23:1. This ratio is comparable to that for humans (28:1), and Eger suggests that it indicates a similarity in the buffering capacities of the salivas of the two species. It further, however, suggests a similarity of action in both, and may indicate a sensitivity to hydronium ion in these caterpillars corresponding to the sour taste in humans.

Salts

The order of relative stimulative efficiencies, as measured by the reciprocals of the normalities of the rejection limens, for the various cations, as chlorides, gives the series: $NH_4^+ = K^+ > Ca^{++} > Na^+ > Li^+$.

This series can be verified further by checking the orders for the twelve individuals to determine in how many cases any specific cation has an equal, greater, or lesser stimulative efficiency than any other cation. Using this method, it is found that K^+ has a greater efficiency than NH_4^+ ($K^+ > NH_4^+$) in 4 individuals, NH_4^+ has greater than K^+ ($K^+ < NH_4^+$) in 3 individuals, and K^+ is equal to NH_4^+ ($K^+ = NH_4^+$) in 5 individuals. This indicates that K^+ and NH_4^+ are equal in stimulative efficiency. Comparing K^+ and NH_4^+ with Na^+, the following orders are noted: $K^+ > Na^+ - 9$, $K^+ = Na^+ - 3$, $K^+ < Na^+ - 0$, and $NH_4^+ > Na^+ - 8$, $NH_4^+ = Na^+ - 4$, $NH_4^+ < Na^+ - 0$. These clearly show that K^+ and NH_4^+ have greater stimulative efficiencies than Na^+, and further confirm their equality. Comparing Na^+ with Li^+, the following are found: $Na^+ > Li^+ - 9$, $Na^+ = Li^+ - 3$, $Na^+ < Li^+ - 0$. Obviously, Na^+ is more stimulating than Li^+. K^+ and NH_4^+ are

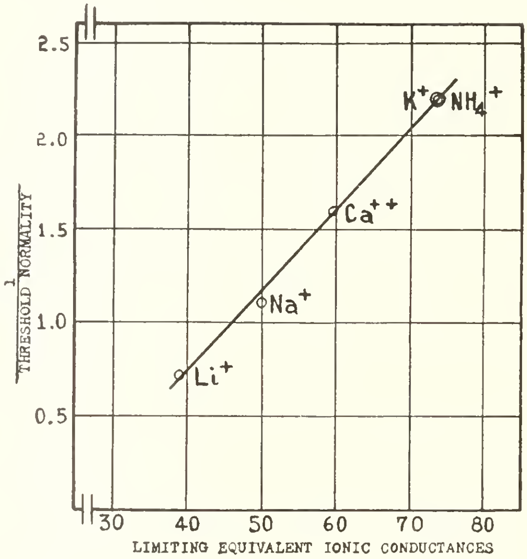

FIGURE 1. Showing the relationship between the stimulative efficiencies of the cations, as chlorides, and their limiting equivalent ionic conductances in Mhos at 25° C. Values for the latter are from Gucker and Meldrum (1942).

found to be more stimulating than Li^+ in all cases, thus verifying their position above Na^+. Ca^{++} shows the greatest variation in position. Comparing with NH_4^+ or K^+, the following are found: K^+ or $NH_4^+ > Ca^{++} - 8$, K^+ or $NH_4^+ = Ca^{++} - 2$, K^+ or $NH_4^+ < Ca^{++} - 2$. Thus K^+ and NH_4^+ have greater stimulative effects than Ca^{++}. Comparing Ca^{++} with Na^+ we find: $Ca^{++} > Na^+ - 9$, $Ca^{++} = Na^+ - 0$, $Ca^{++} < Na^+ - 3$. Ca^{++} is thus more stimulating than Na^+. With Li^+ we find: $Ca^{++} > Li^+ - 11$, $Ca^{++} = Li^+ - 1$, $Ca^{++} < Li^+ - 0$; Ca^{++} is thus more stimulating than Li^+. All of these individual orders considered together indicate the same arrangement as that found using the mean thresholds, namely: $NH_4^+ = K^+ > Ca^{++} > Na^+ > Li^+$.

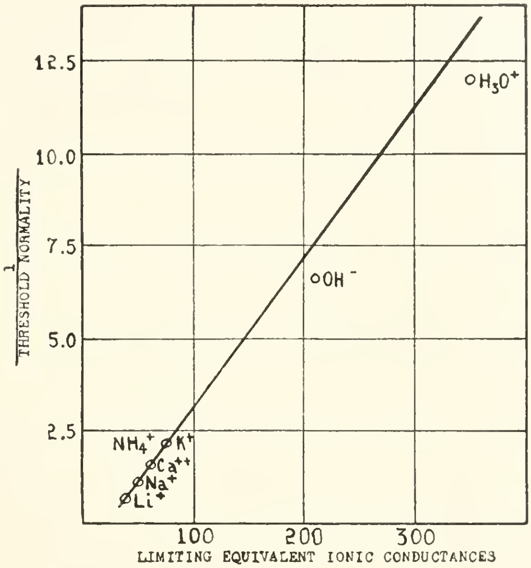

FIGURE 2. Showing the relationship between the stimulative efficiencies and the limiting equivalent ionic conductances in Mhos at 25° C. of certain ions. The slope is taken as determined by the metallic ions. Values for the conductances are from Gucker and Meldrum (1942).

Hopkins (1932) has tabulated the discoveries of numerous investigators concerning stimulative efficiencies of various cations and anions. The order shown in the case of the caterpillars of *Samia cecropia* here used is similar to that discovered for other species of animals. This suggests some basic chemical or physical reason for this order. Hopkins relates the order of cations for the oyster, which he studied, to the atomic weights and atomic mobilities. In the present case, there is no obvious relationship between ionic weights and stimulative efficiences, but Figure 1 shows the close agreement between the stimulative efficiencies of the cations, as determined by the reciprocals of the normalities of the rejection limens, and their limiting equivalent ionic conductances which are directly proportional to the ionic mobilities, in fact, have sometimes been called mobilities. Since the chloride ion is the anion in all cases, it is impossible to determine its relative stimulative effect, and it is, therefore, disregarded in this plot, since, whatever its efficiency, it is constant for all substances tested.

These relationships suggest a common mode of action, and possibly a common modality of taste, for all the cations tested. Since, however, this same order is found for man for K^+, Na^+, and Li^+, and KCl has a taste easily distinguishable from NaCl or LiCl, this conclusion is not warranted without further evidence.

Sodium hydroxide shows a much greater stimulative efficiency than any of the other salts[1] used in this work, thus indicating that the OH^- ion is the critical one in its effectiveness, since some measure of the efficiency of the Na^+ ion is given by the stimulative efficiency of NaCl. Comparison of the data for NaOH and HCl shows that the OH^- ion is much less stimulating than the H_3O^+ ion.

Figure 2 shows the extrapolation of the graph, stimulative efficiency vs. limiting equivalent ionic conductance to include OH^- and H_3O^+. The value for the stimulative efficiency of the OH^- ion is approximated by subtracting from the stimulative efficiency for NaOH the stimulative efficiency for NaCl, assuming thus that most of this is due to the Na^+ ion. This shows that there is a possibility that OH^- and H_3O^+ fall in the same series. That they are apparently lower in stimulative efficiency than is needed for a perfect "fit" in the graph would be expected from the fact that the solutions containing them were mixed with the protoplasm of the leaves and with the saliva of the caterpillars, both of which are buffered. This might mean that there is only an "unacceptable" modality of taste for all these substances, as Eger (1937) suggests, but this cannot be decided without further evidence. The OH^- and H_3O^+ ions, in the case of man, show a high stimulative efficiency when compared with other anions and cations, and stimulation by the H_3O^+ ion, at least, is admitted to be the important factor in the sour taste, as opposed to the salt taste of the metallic chlorides.

In all, these results can only be taken as suggestive and indicative of a need for much more extensive and careful work on the gustatory responses of animals to more than the usual NaCl in testing the salt taste.

SUMMARY

Rejection thresholds for HCl, CH_3COOH, NaOH, NaCl, NH_4Cl, KCl, $CaCl_2$, and LiCl, presented as drops of solutions on leaves of the food plant, were determined for caterpillars of the cecropia moth, *Samia cecropia*. Rejection thresholds for glucose, sucrose, lactose, and strychnine sulfate either do not exist under these conditions, or are higher than the saturation concentrations of solutions of these substances. The lowest threshold of those tested is that for HCl, but CH_3COOH has greater stimulative efficiency when it is compared with HCl at the same pH. The threshold for NaOH is higher than that for HCl, indicating that the OH^- ion is less stimulating than the H_3O^+ ion. The order of stimulative efficiency for the cations, as chlorides, is $NH_4^+ = K^+ > Ca^{++} > Na^+ > Li^+$. This is the order of ionic mobilities to which the stimulative efficiencies seem to be related. No conclusions can be drawn with certainty as yet regarding the modalities of taste for these animals.

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[1] Modern Acid-Base concepts require that NaOH be considered as a salt.

ACKNOWLEDGEMENTS

It is a pleasure to express my appreciation for the many kindnesses shown me during this work by Dr. Nicholas Hyma, Head of the Department of Chemistry, and Dr. Arthur B. Gould, Professor of Chemistry, at this institution. An especial debt is due my wife who aided me in all phases of the work.

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BACKGROUND ILLUMINATION AS A FACTOR IN THE ATTACHMENT OF BARNACLE CYPRIDS

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INTRODUCTION

Various authors, following Visscher (1927), have shown that certain species of barnacles attach in greater numbers to darker surfaces. McDougall (1943), by means of an experiment designed to test the effect of varying degrees of illumination, demonstrated that larvae of *Balanus eburneus* have a tendency to settle most abundantly upon collectors placed in less brightly illuminated areas. Pomerat and Reiner (1942) showed that *Balanus eburneus* larvae attach more readily to black rather than to opal panels when they are exposed in the sea for several days. These investigators also observed that when light is at a minimum, during the hours of darkness, the differences do not occur but that attachments are remarkably similar on light and dark panels. This suggested that attachment to darker panels during daylight is a phototropic response to the contrasting effect of dark surfaces against lighter general surroundings. The purpose of the present study was to investigate whether in fact, contrasting surroundings are effective in promoting attachment. A further objective was the determination of the maximum distance at which the degree of illumination of a background surface is effective in influencing attachment to transparent surfaces.

The experiments were conducted at the Pensacola, Florida laboratory of the U. S. Fish and Wildlife Service by the kind permission of Dr. A. E. Hopkins during the summer months of June, July, and August, 1942. Acknowledgments are also due to Dr. C. M. Pomerat for his generous advice, and to Dr. F. G. Walton Smith, Director of the University of Miami Marine Laboratory, for assistance in preparing the paper and in evaluating the results.

EFFECT OF CONTRASTING BACKGROUNDS

In the experiments designed to determine whether contrasting surroundings influence cyprid attachment, provision was made for base plates of black, transparent, and opal Carrara glass plates 10″ × 12″ in size. Upon each of these were mounted 4″ × 10″ black and opal Carrara glass collecting panels, in such a way as to be surrounded by a 2 inch border of the base panel.

The plates were supported by a wooden frame. The glass bases were arranged one inch apart and the paired black and opal collectors were separated by two inches. Thus, a series of contrasts was offered between the pairs of black and opal plates and the three surrounding borders of black, opal, and transparent glass. The barnacles were counted following attachment upon the collectors, each of which offered an exposed area of 36 sq.in. In the locality where the investigations

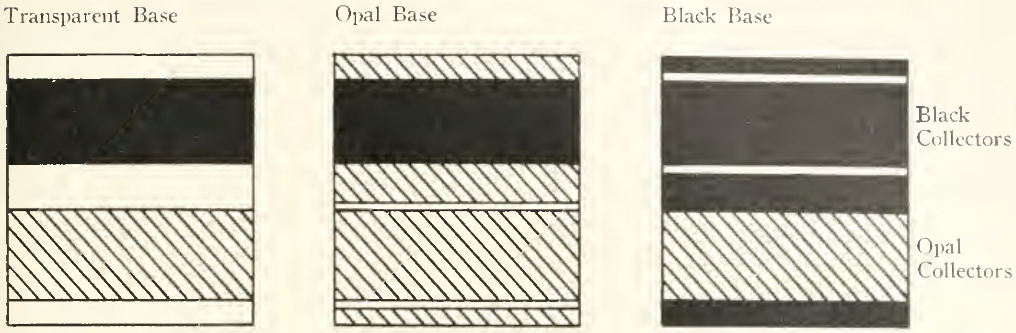

FIGURE 1. Arrangement of black and opal collectors upon black, opal, and transparent bases.

were carried out the adults of *Balanus eburneus* were the barnacles most commonly found, and all attachments were assumed to belong to this species.

The apparatus for the first series of experiments was lowered into the sea with the plates in a vertical position at a mean depth of three feet from the surface. Six successive experiments were conducted with an average duration of 36 hours. At the end of each period the paired plates were removed and washed gently with fresh water to remove salt crystals and silt. Counts were then made of the cyprids and of the metamorphosed barnacles, with the results shown in Table I.

Further experiments were conducted with the plates exposed horizontally, in order to collect attachments on the lower surface. The mean depth of the plates below the surface of the sea was seven feet. Five experiments were conducted,

TABLE I

Barnacles attaching to vertical collectors with borders of varying contrast

Experiment	Date of exposure	Black collector			Opal collector		
		Borders			Borders		
		Transparent	Opal	Black	Transparent	Opal	Black
I	7-2-44	68	67	35	15	17	8
II	7-4-44	300	293	458	249	183	233
III	7-6-44	73	115	59	8	5	7
IV	7-8-44	120	175	121	15	38	35
V	7-10-44	89	97	86	17	7	25
VI	7-12-44	137	152	162	23	23	18
Total		787	899	921	327	273	326
Average		131	150	154	55	46	54

TABLE II

Barnacles attaching to horizontal collectors with borders of varying contrast

Experiment	Date of exposure	Black collector			Opal collector		
		Borders			Borders		
		Transparent	Opal	Black	Transparent	Opal	Black
VII	7–17–44	653	503	703	80	114	171
VIII	7–20–44	757	692	711	94	142	194
IX	7–23–44	582	720	686	142	249	340
X	7–26–44	620	855	701	254	328	437
XI	7–31–44	526	582	564	194	230	303
Total		3138	3352	3365	764	1063	1445
Average		628	670	673	153	213	289

with exposure periods varying from 72 to 120 hours. The results are shown in Table II.

Upon horizontal and vertical black collectors and regardless of the nature of the surroundings, greater attachments occurred upon the black collectors as compared with the opal. The total number of organisms on all black horizontal collectors amounted to 9855 compared with a total of 3272 for opal horizontal collectors, giving a black/opal distribution ratio of 3.0. The total populations on black and opal vertical collectors were 2607 and 926 respectively, giving a ratio of 2.8. These ratios, though greater, qualitatively substantiate the findings of Pomerat and Reiner (1942) whose ratio for attachment numbers upon black and opal collectors was 1.8 (Table III).

TABLE III

Total number of barnacles on black and opal collectors

	Black collectors	Opal collectors	Ratio $\frac{\text{Black collectors}}{\text{Opal collectors}}$
Horizontal	9855	3272	3.0
Vertical	2607	926	2.8

Attachments to black vertical collectors did not vary greatly with the type of surrounding, but were least in the case of the transparent border (Table I). With black collectors, borders of black, transparent and opal, in this order, afford increasing contrasts. Had the contrast been effective, greater attachments should have occurred upon collectors with the opal and transparent borders rather than with the black border. Attachments upon black horizontal collectors also failed to show significant differences when the three different surroundings were employed, although they were least in the case of a transparent border (Table II).

The frequencies of attachment upon vertical opal collectors did not show any correlation with increasing degree of contrast but were similar in all instances (Table I). The horizontal opal collectors gave somewhat different results (Table II). In this series of experiments the greatest attachments were found on the collectors with the black borders. The least numbers, however, were observed on collectors with transparent borders and intermediate attachment frequency occurred with opal borders. In these experiments the least degree of contrast was offered by opal surroundings of similar material to the collectors and the greatest degree by black surroundings which afforded the minimum possibilities of transmitted and reflected light. There was not, therefore, a consistent correlation between the intensity of attachment and the degree of contrast between collector and surroundings.

In an attempt to explain the results, the question of variation in the general intensity of illumination beneath the panels may be considered. The amount of transmitted and reflected light was greatest around horizontal opal collectors with transparent surroundings. The reflected light which alone fell below opal collectors with the opal borders was less intense than the light in the former case and it was under this condition that frequency of attachment was found to be increased. The opal collectors with black borders, which reflected the least light of the three types of surrounding were found to have the greatest attachment. Thus, with the decrease in general illumination beneath opal collectors, there was an increase in attachments, and it appears that attachment frequencies are here related to the degree of shading under the collectors caused by the varying opacity and reflection of the surroundings.

The horizontal black collectors were found to have distinctly greater attachments when combined with a black border. They also showed least attachments with a clear border. The black collectors, therefore showed a correlation, not with contrast but rather with the degree of shading which is at a maximum where both collector and surroundings are black.

In the case of both black and opal vertical collectors, the shading effect of the border is less pronounced, due to the fact that much of the light enters the water at a more or less vertical angle. The hypothesis that general shading stimulates attachment would fit in with the observed facts about attachments to these series. The difference between the amount of light reflected by black or opal collectors is more significant than that reflected by the relatively small borders, and hence accounts for the relatively small differences among attachments to black collectors with different surroundings or among attachments to opal collectors with varied surroundings. The differences between attachments to the two types of collector with each surrounding are relatively greater.

Distance At Which Background Illumination Is Effective

The equipment designed for the question of determining the maximum distance at which black or opal surfaces are effective in stimulating a cyprid to attach consisted of a wooden frame 30″ long, 32″ wide, and 12″ high. This frame supported vertically two transparent glass plates. Behind the first glass plate was mounted a black plastic panel in such a manner that it might be moved to varying distances. The second transparent glass plate without any backing was used as the control. A second series of transparent glass plates at the opposite side of the frame were treated in the same manner with the exception that an opal plastic panel was used instead of a black one. The movable black plastic panel and the movable opal plastic panel were set at increasing distances from the clear panel for successive experiments. Each transparent glass plate from which the barnacles were counted, offered 54 sq.in. of surface area. They were exposed at a mean depth of three feet from the surface for an average duration of 72 hours. In the successive experiments the black and opal plastic movable panels were placed at 2″, 3″, 4″, and 6″ behind the transparent collectors.

The results (Table IV) show a general decline in the attachments to the glass collector as the black movable plate was moved away. The maximum distance at which it influenced attachment, as determined by comparison with the control, was between four and six inches. The same general decline in population was noted for the opal plates although the maximum influencing range was greater than six inches. Since attachment is influenced by the movable panels at a distance, it is obvious that the light reflection at the actual collecting surface is not the principal stimulating factor. The facts are more readily explained by assuming that the intensity of general illumination in the vicinity of the collector is the important factor and that where this is reduced by the proximity of black backplates greater attachment occurs. Decreasing distances of the opal backplate, while giving a superficial appearance of increased light intensity, probably succeed in blocking the light passing through the collecting panel. Thus increased attachment under these conditions may still be due to decreased general illumination.

TABLE IV

Attachment of barnacles to transparent panels with movable backgrounds

Distance of background from collector	Movable black	Control transparent	Ratio $\frac{\text{Movable}}{\text{Control}}$	Movable opal	Control transparent	Ratio $\frac{\text{Movable}}{\text{Control}}$
2 inches	2998	778	3.9	1258	511	2.5
3 inches	803	355	2.3	835	336	2.5
4 inches	3759	1707	2.2	3686	2806	1.3
6 inches	458	453	1.0	906	780	1.2

These general conclusions agree with the results of the contrasting background experiments described in the first part of the paper where a relationship appeared to exist between the number of attachments and the amount of shading caused by the type of background employed. The decreased intensity of illumination in the general vicinity of the collector, as opposed to the conditions of illumination immediately upon the collector itself may increase attachment by causing a response in the cyprid. This response is in the nature of a conditioning towards subsequent attachment, or the development of a physiological state favoring a subsequent attachment response to a contact stimulus. The possibility of a tropism is not supported by the contrast experiments.

In the light of the above conclusions it may be interesting to recall the experiments of Schallek (1943) and Whitney (1941) who maintain that directional light is not to any great degree present under natural aquatic conditions but that conditions of diffuse light almost invariably predominate. This diffuse light is a naturally occurring phenomenon, whereas light from any one direction may not predominate sufficiently for a tropism to occur.

SUMMARY

1. Experiments were conducted to determine the effect of contrasting surroundings upon the frequency of attachment of *Balanus eburneus* larvae to opaque black and opal glass collecting surfaces. Further experiments were carried out to determine to what extent the number of attachments of transparent collectors was influenced by black and opal backgrounds placed at varying distances.

2. Greater numbers of attachments occurred upon the under side of horizontal rather than vertical, and upon black rather than opal collectors, thus confirming the observations of previous authors.

3. No correlation was found between the degree of contrast shown in the collector and surroundings, and the frequency of attachments.

4. Both black and opal surfaces were found to increase frequency of attachment when placed behind transparent collectors up to distances of six inches.

5. A definite dependence was found to exist between the frequency of attachment and a decrease in the intensity of general illumination in the area immediately beneath horizontal opal collectors. Similarly, the influence of movable backgrounds appeared to be in the nature of a shadow effect. It is suggested that "shading" acts as a stimulus which brings about favorable physiological conditions for the subsequent attachment of barnacle larvae and that the amount of light reflected from the collecting surface is only important insofar as it affects the general "shading" in the vicinity.

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THE CONDITIONS THAT LEAD TO NORMAL OR ABNORMAL DEVELOPMENT OF CIONA

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In earlier papers I have discussed the problem of normal versus abnormal development of cross-fertilized eggs of Ciona. It was shown that external conditions sometimes influence the result; at other times it was not apparent that the abnormal development was due to such conditions. However, by a critical series of experiments evidence was found that the eggs are very sensitive to what may be called the "cleanliness" of the dishes, i.e. to either chemical or organic contamination. Further experiments have been made to find out whether internal factors may also be concerned with abnormal development of the eggs.

Ciona is a particularly favorable type for study of this problem. The eggs, as soon as they are mature, leave the ovary and accumulate for 24 hours or longer in the oviduct. The germinal vesicle has disappeared and the polar spindle comes to lie at the pole of the egg. The polar bodies are not given off until a spermatozoon enters the egg, i.e. after the eggs have been set free in the sea water. This may happen every 24 hours or be delayed for several days. Spawning occurs early in the morning. If not ejected at the regular time the eggs may accumulate in the oviduct in very large numbers. When the animals are brought into the laboratory and kept in running sea water, or in sea water that is aerated by a stream of air bubbles, the Cionas may hold back their eggs for several days. It has been shown that these delayed eggs may give rise to normal embryos, but it has not been shown clearly that the percentage of abnormals may not be increased. Fresh eggs removed from the oviduct and kept for as long as 24 hours in covered dishes of shallow sea water can be cross fertilized and may give normal embryos.

The sperm are matured in the tubes of the testes that ramify over the walls of the intestine in the region of the outlet of the ovary. The ripe sperms pass into the long sperm duct that runs parallel to the oviduct. Only ripe sperms are present in the sperm duct, and some of them are set free when the eggs are ejected. When the eggs are collected by puncturing the oviduct, only mature eggs are set free, and likewise only ripe sperm comes out when the sperm duct is cut across. In most other marine animals, whose eggs are used for experimental purposes, one is apt to get ripe and unripe eggs when the ovary is opened. The uniformity of the eggs from the oviduct of Ciona simplifies the experiment.

In another respect, also, Ciona offers favorable material for a study of normal versus abnormal development. It is well known that polyspermy often plays a significant note in laboratory experiments, unless it is carefully guarded against. In Ciona, polyspermy also occurs after cross-fertilization, but is very rare and will account for only a small percentage of abnormality. It can easily be detected at the two-cell stage when the egg divides into four or more parts if polyspermic.

Even in cases where much abnormality occurs, I have found, by watching the cleavage, that polyspermy was not the main agent in the results.

Concerning the kind of abnormalities that occur, not much can be said. For convenience I have recognized three classes; (1) Normals, (2) Abnormals, and (3) Bent (or twisted) embryos. The abnormals are those that have not gone beyond an early stage of development. They are usually in the egg membrane and are spherical with one or two black "eye" specks. The twisted or bent are later stages in development, usually out of the egg membrane. The tail is present and crooked or bent, sometimes still coiled loosely or irregularly about the body. There are overlaps between the three different types. All three are generally found in the same dish, but the external or internal conditions that produce each kind are not known. It is safe to assume, however, that polyspermic eggs do not pass beyond the earliest stages of development, but external conditions may also stop the development in early stages.

Normal and Abnormal Development in Reciprocal Crosses

To test to what extent the ratios of normal to abnormal development are due to intrinsic (genetic?) factors or due to environmental conditions, further experiments were made in May 1944. Not only were reciprocal crosses made, but samples of the same eggs were crossed with three different sperm suspensions.

TABLE I

The percentage of normal embryos resulting from reciprocal crosses. The small numbers under the percentages represent, from left to right, the numbers of normal, bent, and abnormal embryos. The bent embryos have been left out of account in calculating the percentages.

c sp	*b sp*	*a sp*	
0 0 0 148	93 126 0 8	8 12 42 132	*D eggs*
70 67 9 28	89 49 2 6	99 128 0 1	*E eggs*
59 73 33 49	96 310 1 12	49 51 7 54	*F eggs*
f sp	*e sp*	*d sp*	
0 0 0 37	3 1 2 28	52 10 22 9	*A eggs*
14 10 12 62	2 1 0 37	72 43 11 16	*B eggs*
16 22 29 116	10 11 23 96	0 0 0 177	*C eggs*

Some of the eggs of A were distributed in three dishes (horizontal lines, Table I), also some of the eggs of B and C, each to three dishes. These were cross-fertilized with sperm of d, e and f. Two drops of sperm were added to each dish, and the normal, bent, and abnormal embryos recorded after 24 hours.

The eggs of D, E, and F had been put into three dishes each (horizontal lines) before the sperm of the same animals, d, e, and f, had been removed to cross-fertilize the eggs of A, B, and C. Then two drops of the sperm suspension of a, b, and c were added to the D, E, and F eggs. These crosses are the reciprocals of the preceding ones.

In calculating the percentages, the bent embryos were left out of account. Most of them were so nearly fully developed that they might be classified with the normal, rather than with the abnormals. The bent embryos overlapped those abnormal embryos that were fairly well developed. The bent embryos would not have affected the percentages to any great extent if half of them had been combined with the normals and half with the abnormals.

As shown in Table I the percentages of normal embryos resulting from crossing A eggs by d sperm, e sperm and f sperm are very different (52, 3, 0%). This holds also for B eggs and C eggs by the same sperm. In other words, the results are different for the different combinations, and not consistent either for the eggs or for the sperm.

The reciprocal crosses are shown above in the same Table I. Comparing them, there is seen to be no agreement in the percentages. In other words, the reciprocals are no more likely to be alike than other crosses. Before discussing these results, another similar experiment will be described that was made the next day with Cionas brought to Pasadena (Table II).

This experiment was carried out in the same way as the last, except that in addition to the reciprocals, three more dishes of A, B, and C eggs were set out and crossed with sperm from three other Cionas, viz. g, h, and i. These serve to give further data for the same eggs with different sperm. The reciprocals of g, h, i were not made. Comparing the reciprocals of the first three tests, D, E, and F, each set are more like each other than are the sperm tests a, b, and c, and also more like each other than in the first experiment, but still not the same inter se. The F eggs were much fewer than the others and produced relatively much smaller numbers of normals.

A comparison of the three kinds of eggs, A, B, and C each with the six kinds of sperm, d–i, shows that there are wide differences in the percentages (Table II). Since the environment was as nearly the same as possible, it may seem to follow that the differences are due to internal factors of some kind, possibly genetic in origin.

Why are the reciprocals so often different? The diploid eggs are presumably all genetically alike in each Ciona before fertilization, but after the polar bodies are extruded, the eggs become haploid, and should give as many kinds of eggs as there are combinations of the six pairs of chromosomes (excluding the possibility of a larger number due to crossing over). If amongst these combinations there are certain combinations that might give abnormal development—lethals, in fact,—it might seem that the chances are about the same in the cross and in its reciprocal. In other words, even with many lethal factor combinations, any differences in reciprocal ratios would only be due to chance, unless the cytoplasm of the diploid egg is involved in the situation.

There are several more or less probable hypotheses that suggest themselves, and some of these may or may not have some connection with the theory of self-sterility in Ciona; for, if certain classes should be eliminated, those that remain and produce normal tadpoles might, theoretically, be the ones that carry factors for self-sterility.

An examination of the data from the dishes of the same eggs (horizontal lines; Table II), each fertilized by different sperm, show very great differences in most cases in the percentages of normals to abnormals. Occasionally, however, a set gives very low values (Table I, F eggs by (a), (b), (c) sperm) or very high ratios (Table II, E eggs by (a), (b), (c) sperm. Also an examination of the ratios of the same sperm used to fertilize three different lots of eggs shows sometimes high percentages in all three dishes (Table II, (d) sperm by A, B, C eggs) and at other times low percentages in all three dishes (Table II, (g) sperm by A, B, C eggs). On the other hand there are cases where each of the three sets of eggs, and also each of the three sets of sperm, show great differences in the ratios.

If the data are significant, i.e., if the differences are not due to infection in the dishes, these results show that the outcome is due neither to the eggs alone nor to the sperm alone, but to the combinations found at the time of fertilization.

TABLE II

The percentages of normal embryos resulting from reciprocal and one way crosses. The small numbers under the percentages represent, from left to right, the numbers of normal, bent, and abnormal embryos. The bent embryos have been left out of account in calculating the percentages.

			c sp	*b sp*	*a sp*	
			97 71 2 1	**76** 287 8 35	**91** 152 3 14	*D eggs*
			98 303 12 6	**95** 256 3 9	**98** 289 11 5	*E eggs*
			3 1 0 32	**2** 1 0 48	**0** 0 0 34	*F eggs*
i sp	*h sp*	*g sp*	*f sp*	*e sp*	*d sp*	
99 319 0 3	**37** 69 34 118	**3** 8 17 258	**99** 236 1 1	**96** 225 0 9	**82** 133 26 29	*A eggs*
2.8 9 65 280	**0** 0 8 286	**0.2** 1 0 403	**76** 360 72 112	**58** 136 24 85	**97** 400 6 14	*B eggs*
0 0 0 101	**50** 36 32 35	**12** 1 8 7	**47** 30 29 33	**91** 200 1 24	**88** 82 7 22	*C eggs*

In this connection there are some further considerations to be taken into account. The cytoplasm of the eggs has developed under the influence of the diploid set of chromosomes. If the constitution of this cytoplasm determines whether the development will be normal or abnormal, then the eggs of one individual might be expected to develop in the same way irrespective of what kind of sperm fertilizes the eggs. On the other hand, after the extrusion of the polar bodies, the haploid egg nucleus may have a very different make-up from the original diploid nucleus. But it is not likely that this haploid nucleus can affect or change the constitution of the cytoplasm in the very short time before it combines with the sperm nucleus to form a new diploid nucleus, whose products carry on throughout the following development. From the evidence discussed above it might seem, therefore, that whether the development is normal or abnormal will depend on whether the combination of the two haploid genetic chromosome groups are harmonious or not in their action on the cytoplasm. If the cytoplasm is indifferent, then reciprocal crosses should give the same ratios, but since they do not do so, it may seem to follow that the cytoplasm, which has developed under the influence of the diploid egg nucleus, is one of the factors in determining whether normal or abnormal development takes place. This conclusion would mean that certain kinds of combinations are being continually eliminated. But it would not necessarily mean that these have anything to do with cross and self-fertilization of the combinations that survive. However, experiments that will be described later make it probable that the differences in the dishes reviewed in this experimnt are due to external factors, such as toxic infection, and are not due to inherited cytoplasmic differences in the eggs.

Test for Polyspermy and Abnormal Development

Five new sets of experiments were made (Table III) to test whether the abnormals that appear frequently are due to polyspermy, although it had often been recorded that the division of the eggs at once into four (or more) cells only very rarely occurs. Forty, 20 or 2 drops of sperm suspension were added to eggs in Syracuse dishes. After one hour the supernatant fluid was changed to fresh sea water. Reciprocal crosses were made in all cases. As the table shows, Table III, the relative number of normals to abnormals was no lower after 40 drops than after 2 drops of sperm suspension. Evidently polyspermy is not the cause of abnormal development. There are several striking exceptions to the general results. One (June 18–19, 1944), A^1–b^1, gave after 2 drops almost all abnormals (328), although after 40 or 20 drops nearly all were normal. Again (June 19–20, A^2–b^2, in one case after 20 drops all were abnormal (295); and in the reciprocal after 40 drops all were abnormal (199), although after 20 and 2 drops nearly all were normal. In another test (June 18–19), A^3–b^3, normals greatly predominated. In another (June 18–19), A^4–b^4, all were abnormal after 20 drops, but normal after 40 and after 2 drops sperm suspension. In another lot (June 17–18), A^5–b^5 and B^5–a^5, there were two cases where there were more abnormals than normals. These apparently contradictory results can only be explained on the assumption that the dishes were in some way responsible for the abnormal developments.

Delay in Fertilization and Abnormal Development

Whether or not a delay in fertilization causes abnormality after eggs and sperm have been kept in sea water was tested. The results are recorded in Table IV. The eggs of three individuals were distributed amongst six dishes. Then the eggs of A were fertilized by 10 drops of the sperm suspension of b and c at once, and after an hour and two hours. Similarly the eggs of B were fertilized by the sperm of a and c; and for the eggs of C by the sperm of a and b.

In A by b normals were produced by fertilizing at once, and after one hour, but nearly all abnormals after two hours. This is true also for A by c, but B by a and B by c and C by b gave more normals after two hours. C by a gave normals after one hour, but no fertilization after 2 hours. Perhaps the most striking results are A by b and A by c that gave normals at once and after a delay of one hour, but abnormals after two hours, but this did not happen in two other cases.

Either the sperm in the two latter cases was less affected by the delay, or there was some kind of difference in the dishes themselves. The irregularities in this experiment could only be accounted for on the assumption of infection in some of the dishes. The test was repeated (Oct. 14, 1944) with the eggs of new Cionas at Corona Del Mar, and transferred to Pasadena in autoclaved flasks and stenders. Five of the lots fertilized at once, A by b, gave 99.9 per cent normals; one (B by c) gave 75 per cent normals. After 2 hours A by b gave 99.9; A by c, one normal and the rest unfertilized; A by c gave 0.5 per cent normal and the rest unfertilized.

TABLE III

The effect of density of insemination in reciprocal crosses on the proportions of normal tadpoles, bent embryos, and abnormal embryos.

(June 18–19, 1944)

A^1 by b^1	Tad	Bent	Abn.	B^1 by a^1	Tad	Bent	Abn.
40 drops	405	0	0	40 drops	406	6	17
20 drops	443	0	2	20 drops	471	3	13
2 drops	3	40	328	2 drops	364	8	4

(June 19–20, 1944)

A^2 by b^2	Tad	Bent	Abn.	B^2 by a^2	Tad	Bent	Abn.
40 drops	122	21	39	40 drops	0	0	199
20 drops	0	0	295	20 drops	149	12	7
2 drops	351	0	0	2 drops	91	0	0

(June 18–19, 1944)

A^3 by b^3	Tad	Bent	Abn.	B^3 by a^3	Tad	Bent	Abn.
40 drops	211	5	0	40 drops	205	10	21
20 drops	268	7	17	20 drops	101	23	26
2 drops	148	14	7	2 drops	231	1	3

(June 18–19, 1944)

A^4 by b^4	Tad	Bent	Abn.	B^4 by a^4	Tad	Bent	Abn.
40 drops	230	0	0	40 drops	225	24	37
20 drops	0	0	263	20 drops	330	0	1
2 drops	266	0	0	2 drops	218	19	38

(June 17–18, 1944)

A^5 by b^5	Tad	Bent	Abn.	B^5 by a^5	Tad	Bent	Abn.
20 drops	377	6	7	20 drops	607	13	31
10 drops	293	3	1	10 drops	684	2	12
2 drops	45	43	116	2 drops	191	35	278

Table IV

The effect of delaying insemination in reciprocal crosses on the proportions of normal, bent, and abnormal embryos, and unfertilized eggs.

		N	Bent	Abn.	Unfert.			N	Bent	Abn.	Unfert
A by b	at once	504	0	7	6	B by a	at once	66	13	232	93
	1 hr.	343	0	1	0		1 hr.	131	2	84	98
	2 hrs.	3	5	390	25		2 hrs.	204	0	0	179
A by c	at once	428	0	20	27	C by a	at once	317	7	48	11
	1 hr.	464	0	9	27		1 hr.	304	1	11	12
	2 hrs.	14	30	364	4		2 hrs.	0	0	0	498
B by c	at once	763	8	39	20	C by b	at once	721	0	15	5
	1 hr.	483	14	63	4		1 hr.	870	0	8	6
	2 hrs.	888	2	10	7		2 hrs.	910	1	1	157

After 3 hours A by b gave 95 per cent normals and 5 per cent unfertilized; A by c gave 99 per cent normals and 1 per cent unfertilized; B by c were all unfertilized. Reciprocally B by a at once was, by oversight, not fertilized; but after 2 hours 99.8 per cent, and after 3 hours 100 per cent were normals. C by a at once gave 99.9 per cent normals; after 2 hours 99.9 per cent normals; and after 3 hours 100 per cent normals. C by b at once gave 100 per cent normals; after two hours 99.9; and after three hours none were fertilized.

Another test of the same sort gave much the same kind of result. The dishes had been boiled. The percentages of normals was not so high, but there was no evidence of more abnormals after one or 2 hours.

These tests make it quite clear that delay in fertilization up to 3 hours does not in itself cause abnormal development. The irregularities shown in Table IV may safely be ascribed to infection of the dishes. In fact other tests have shown that eggs may be kept in sea water for 24 hours, and, if fertilized with fresh sperm produce normal tadpoles.

Eggs Fertilized En Masse and Distributed at Two-Cell Stage to Separate Small Dishes

The exceptional cases, in which most or all of the eggs developed abnormally whereas others treated in the same way developed normally, suggested that some environmental conditions were responsible for the abnormals. The following experiments were made to further test this suggestion. Eggs were collected from the oviduct in large clusters, and then drawn up by a pipette and transferred to finger bowls with about 80 cc. sea water. They were cross-fertilized with 10 to 20 drops of sperm suspension. After about one hour, when the two-cell stage was reached, about 100 were picked out with a pipette and put into several small dishes or flasks where they remained for about 24 hours, and were then classified. The eggs in the two-cell stage should be nearly alike on the whole, and any difference observed should be environmental. As shown in Table V, there were found some dishes that gave a high percentage of abnormals. It would seem to follow that the differences are due to the environment rather than to internal factors. It is especially to be noted that the eggs left in the finger bowls with more water generally gave nearly 100 per cent normals. The larger volume of water would be expected to dilute any contamination present in the larger dish or introduced with the eggs.

Again, a few eggs (about 100) in the two-cell stage were picked up with a pipette (2 or 3 drops) and each lot put into one of five small flasks, and into five jars with 10 cc. sea water. The flasks were stoppered and the jars had screw tops put on them to prevent evaporation. After twenty hours a few drops of formalin were added to each, and the condition of the embryos examined. Similarly the eggs of individual B were crossed by sperm of a. No counts were made of the 20 lots since practically all dishes contained either 99 or 100 per cent normal tadpoles, except one flask that had 38 tadpoles, 48 bent tadpoles, and 21 abnormal embryos. There was no evident differences between this dish and the others.

TABLE V

The effect of different glassware on the proportion of normal, bent, and abnormal embryos. Each of the two reciprocal crosses was made in a single finger bowl, and eggs in the two cell stage were pipetted into separate small dishes or flasks, in which they remained for about 24 hours.

A by b	N	Bent	Ab	B by a	N	Bent	Ab
1	101	0	3	1	0	0	271
2	103	0	0	2	150	0	0
3	88	2	6	3	0	0	176
4	164	2	2	4	125	1	1
5	29	short 62 bent	4	5	12	56	93
6	15	3	123	6	291	2	5
7	123	7	5	7	75	12	0
8	0	0	165	8	197	6	4
9	100	1	6	9	4	short 121	0
10	148	0	4	10	8	short 68	bent 14

Another experiment, similar to the last, with fresh Cionas was made (Aug. 12, 1944). Five Syracuse dishes (12 cc.) were used for some eggs, 5 Stender dishes for other eggs, and 5 small flasks for others. The dishes were left at Corona del Mar, and formalin added to each after 20 hours. Reciprocal crosses B by a were made. The fertilized eggs, not used, were kept in finger bowls (covered) in about 80 cc. sea water. A by b in the finger bowls gave 95 per cent normals and 5 per cent abnormals. B by a gave 99 per cent normals. The records of the smaller dishes are given in Table VI. It is noticeable that while normals greatly predominated, especially in B by a, there are three striking exceptions where abnormals predominate, and other where a good many abnormals were present. Since the controls—the original larger finger bowls—gave 95 and 98 per cent normals, the exceptional cases in the smaller dishes must have been due to contamination of some kind.

TABLE VI

The effect of different glassware on the proportion of normal, bent, and abnormal embryos resulting from two reciprocal crosses.

		A by b			B by a		
		N	Bent	Ab	N	Bent	Abn
Syracuse	1	0	20	213	61	30	93
	2	121	9	12	115	22	10
	3	112	5	19	52	16	84
	4	100	24	6	157	0	7
	5	87	2	7	107	15	12
Stender	1	51	15	44	56	3	5
	2	80	26	24	188	0	1
	3	1	29	152	132	2	4
	4	81	2	15	134	1	3
	5	142	8	25	128	6	24
Flask	1	58	7	11	35	0	1
	2	89	2	14	77	—	6
	3	193	1	12	53	2	5
	4	72	3	17	86	1	0
	5	71	11	29	123	1	4

Another set of 20 tests was made with Syracuse dishes. The Cionas had been collected the day before and brought to Pasadena. They were in excellent condition with many stored eggs and much sperm. The eggs were inseminated with 30 drops of sperm in finger bowls of 80 cc. sea water. At the two-cell stage about 100 eggs were transferred to Syracuse dishes. Most of the dishes contained a high percentage of normals, but there were six dishes that contained nearly all abnormals, and one that contained a large excess of bent and short embryos.

In the same lot, there were three dishes of A by b left uncovered that gave 12 bent and 111 abnormal embryos; one half covered that gave 3 normals, and 15 bent; and one ⅞ covered that gave 153 normals and 1 abnormal. The three similar reciprocals, B by c, gave nearly all normals. Since evaporation must have been greater in those not covered, and partly covered dishes, than in the covered dishes, and since some of these gave normal, it does not seem probable that enough evaporation takes place in covered Syracuse dishes to cause abnormal development (see below). The large number of eggs kept in the finger bowl gave nearly all normals.

Another experiment was made in the same way (Aug. 21, 1944). Cross-fertilized in finger bowls, the eggs in the two-cell stage were distributed in eight Stender dishes. Five lots gave nearly all normals, but one gave all abnormals, and two gave more abnormals than normals. In the large number of eggs left over in the two finger bowls, one gave 100 per cent normals and the other 99.9 per cent normals. Obviously the conditions in three of the Stender dishes were unfavorable.

Effect of Evaporation of the Sea Water on Development

One of the possible influences that cause abnormal development might be evaporation of the sea water from the covered Syracuse dishes. To test this, 10 cc. of sea water was evaporated in the dishes to a slight extent before the eggs, that had been fertilized in a large volume of water, were transferred to the concentrated sea water (Oct. 16, 1944). As a control, two autoclaved dishes that had been covered, but their volumes reduced only to $9\frac{7}{10}$ and $9\frac{5}{10}$ cc. gave 100 per cent normals. Another dish, where the water had evaporated to $7\frac{9}{10}$ cc., gave all twisted and bent embryos. In another, evaporated to 7 cc., the eggs had remained in 2 cells after 24 hours. In general the water in a covered Syracuse dish is reduced during 24 hours by about $\frac{3}{10}$ to $\frac{5}{10}$ cc. As shown by these and other tests this amount of reduction does not interfere with normal development. Hence, the irregularities observed in the earlier experiments were not due to concentration of the sea water.

Sterilization of Dishes by HCl

Several tests in duplicate were made. In one (A by b, Sept. 3, 1944) the dishes had been sterilized with weak HCl and washed in distilled water and dried. There were eight that gave 95 to 100 normals, but two gave 100 per cent abnormals. Reciprocally, (B by a) there were six that gave normals (90 to 100 per cent) and four that gave mostly abnormals. The high frequency of dishes with abnormals may seem to be due to the acid, but if any was left after washing, it should have evaporated when the dishes were dried (24 hours). Many eggs were left in the finger bowls, and A by b and B by a each gave 100 per cent normals. Whatever the cause of occasional abnormal development, it appears that it was not removed from all the dishes by the HCl treatment. Either the HCl itself was not entirely removed by washing, or else something else was left behind after the treatment that was later the basis of contamination (infection) of the dishes. The result is in accord with earlier experiments of the same sort (*Biol. Bull.*, 80, 1941, pages 348–349).

Sterilization of Dishes by Heat

The records of the preceding experiments show almost without exception, one or more similarly treated dishes with abnormal embryos, while the rest contain only, or largely, normals. Such exceptions occur even when the eggs have been transferred to the smaller dishes at the two- or four-cell stage. The result suggests contamination or infection of some lots. Therefore, a new set of 10 duplicated lots and 10 reciprocals were tested in dishes that had been autoclaved (120° C.). The eggs were collected from the oviduct in such a dish and transferred to a large flask (also autoclaved) and there cross-fertilized. At the two-cell stage, 50 to 100 eggs were transferred to five flasks and five Stenders each, 20 in all. After 24 hours, A by b gave practically all normals, i.e. 85 to 100 per cent. The reciprocals all gave 100 per cent, except one lot that had 50 per cent somewhat bent tadpoles and 50 per cent normals. The eggs left over in the large flasks (A by b) gave 99 per cent normals and one per cent bent, about two thousand in all. In the reciprocal, there were about one thousand normal tadpoles.

Another experiment was made the next day with Cionas brought to Pasadena; 20 dishes were prepared as above (autoclaved). The eggs were kept in Syracuse dishes. They gave 99.9 per cent normals. The many eggs (about two thousand) left in a large beaker (sterilized) also gave 99.9 per cent normals.

A third experiment of the same kind in which the dishes had been boiled for a short time instead of autoclaved, gave nearly the same results. Most of the dishes. A by b. (Syracuse) gave from 95 to 100 per cent normals; only one had 75 per cent normals and 25 per cent abnormals. The reciprocals (B by a) gave five 90 to 98 per cent normals; four, 80 per cent, and one, 75 per cent normals. One (A by b) of the large finger bowls (80 cc.) gave 99.5 per cent normals and 0.5 per cent bent; the other gave only 5 normals, 1 bent and 94 abnormals. It is evident that this finger bowl (B by a) was contaminated, but the eggs that were removed from it at the two-cell stage and transferred to the ten smaller autoclaved dishes with 10 cc. fresh sea water gave 80 to 95 per cent normals.

These sixty tests are very convincing that the cause of abnormal development is due to some contaminating agent present in the dishes, or that develops there (bacterial action). Washing the dishes in tap water and even rinsing them in distilled water does not remove the source of the contamination, while autoclaving the dishes is effective. It should be recalled that in most of the earlier experiments the embryos after 24 hours were killed in weak formalin and the dishes allowed to stand for several hours or days, before washing them again in tap water. They were then dried for a day or longer. Whether the formalin combined with some organic matter in the dishes, or whether the organic matter alone is responsible for the contamination is not shown by these experiments.

Another test was made (September 30–October 1, 1944) with fresh Ciona at Corona del Mar. The eggs were brought to Pasadena in autoclaved flasks and Stenders. Eggs had been fertilized in large finger bowls (80 cc.), and transferred in an early cleavage stage to 5 flasks and 5 stenders, 20 in all. Only normal tadpoles developed. Again, a similar experiment was carried out at Pasadena (Oct. 1–2), but the eggs were transferred to 20 Syracuse dishes that had not been autoclaved since the last time they were used. They gave 99 per cent normals in all, but 5 dishes (out of 20) gave 95 per cent. These two tests corroborated the conclusion that in clean dishes nearly 100 per cent are normal tadpoles. The very small percentage of abnormals may be due to polyspermy, or to other defects in the cleavage.

A different test was carried out as follows (Oct. 1, 1944). A set of 20 dishes was made up as above, using Syracuse dishes that had been washed, but not autoclaved. There were 14 that gave 95 per cent to 100 per cent normals; 4 that gave only abnormals and one that gave 80 per cent normals. The 4 that gave abnormals were carried one step further. The water was drawn off and put into an autoclaved Stender. To the original Syracuse dishes, 10 cc. of fresh sea water was added. Eggs of another individual, in the two cell stage were added to each. After 22 hours all of the Stenders had only abnormal embryos. Evidently the water had been fouled in some way. The six original dishes with fresh water gave 95 per cent normals, 5 per cent abnormals; 50 per cent normals; 100 per cent abnormals; 100 per cent normals; 95 per cent normals; and 80 per cent normals. These eggs did much better in the old dishes with fresh sea water than in the old sea water in a fresh dish.

As a control test, six of the original (Syracuse) dishes that had given only normals were treated in the same way. The six original dishes with fresh sea water (10 cc.) gave all normals. The old sea water from 4 dishes transferred to Stenders gave in one dish normals, another gave abnormals, and another 50 normals and 50 abnormals. More normals resulted than when the original dishes that gave only abnormals were tested. These results are consistent with the assumption that abnormal development is due to toxic material that develops in some of the dishes. It does not seem to be due to the eggs themselves, but to some foreign material present in some of the dishes. Ordinary washing does not remove the material, but sterilization of the dishes does remove it. If the toxic material is due to bacteria left in the dishes, the protein or other substance on which the bacteria grow may be due either to some substance left in the dishes, or to organic material that goes over with the eggs or the sperm.

SUMMARY

Eggs of Ciona that develop in covered Syracuse dishes produce as a rule normal tadpoles in the course of 24 hours, but occasionally only abnormal embryos develop, or both normal and abnormal in the same dish.

Reciprocal crosses sometimes give very different proportions of normals and abnormals, but the relations are often very inconsistent. The results cannot be ascribed to the eggs alone or to the sperm or to their combinations. Even if an attempt is made to refer the outcome of the reciprocal crosses to the cytoplasm of the egg, that has developed under the influence of the diploid nucleus of the egg, still the differences cannot be satisfactorily accounted for.

Polyspermy can account for only a very small percentage of abnormal development, probably not more than half of one per cent.

Delay in fertilizing the eggs does not cause abnormal development. Delay in using the sperm suspension does not cause abnormals.

When the eggs of one individual are fertilized by the same amount of sperm suspension of six other individuals, the outcome may be normals in each dish or normals and abnormals, or occasionally, all are abnormals. When the sperm of one individual is used to fertilize the eggs of six others, the same kind of results may happen.

In order to make the samples of eggs as nearly alike as possible, they were first fertilized in a large volume of water, and, when in the two-cell stage, a few were transferred to several small Syracuse dishes. The same kind of irregularities appeared, which cannot be due to chance selection of different eggs, for the original eggs left in the large amount of water generally gave more than 95 per cent normals.

Therefore, several kinds of experiments were made to find out whether differences in the small dishes will account for the occasional, but persistent, appearance of abnormals. (1) Dirt or impurities in the sea water from the tap was excluded. (2) Evaporation of the sea water in the covered Syracuse dishes was not enough to cause abnormal development. (3) Ordinary washing of the dishes, even rinsing them in distilled water did not remove the cause of abnormal development in some of the dishes. (4) But sterilizing the dishes in an autoclave removed the cause of most of the irregularities.

It follows that the cause of the exceptional cases of abnormals is due to some contamination that remains in the dishes after washing them in fresh tap water. Since the contamination does not affect the early development of eggs (cleavage), but only later stages, it must be putrefactive in origin; and since it is removed by autoclaving the dishes, it is probably bacterial or some sort of organic contamination.

The eggs come to rest on the bottom of the dishes in a few minutes where they remain until the tadpole emerges, hence local differences may affect the development and account for those cases where both normal and abnormal development takes place in the same dish.

The discovery of the cause of the occasional abnormal development removes the possibility that it is due to genetic factors, hence is not concerned with the self-sterility of Ciona.

REGULAR OCCURRENCE OF HETEROPLOIDY IN A GROUP OF PENTATOMIDAE (HEMIPTERA)

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The testis of all pentatomid Hemiptera is composed of lobes which are constant in number for any given species. In some species the size of the spermatocyte cells and sperms may vary in the different lobes, but in any one species there is a characteristic size for each lobe. In other words, if the first lobe in one specimen produces unusually large spermatocytes, the corresponding lobe in other specimens of that species will also show such large spermatocytes. This peculiarity was first discovered by Montgomery (1898), who described it in some detail in a later paper (1910). The phenomenon was called "polymegaly" by Bowen (1920, 1922a, 1922b) who in his exact analysis laid special stress on the processes of sperm formation. He found that certain cytoplasmic elements varied in amount proportionally to the size of the cells, but that the size of the chromosomes varied little or not at all in the different lobes. Normal sperms were formed from all lobes, and differences in the shape of the head arose only because there was greater elongation in some lobes.

The conditions that are basic to this constant variation are therefore subject to thoroughly regulated processes in any given species. They involve no mitotic irregularities and the occurrence of nondisjunction, multipolar spindles and failure of cytoplasmic division, which of course are found here as in other animals and plants, always seem to result from physiological accidents that are sporadic and in no way correlated with polymegaly.

It is therefore of some interest to record a series of cases among the Pentatomidae in which mitotic disturbances occur regularly in a certain lobe of the testis and affect all the cells of that lobe. Moreover such irregularity, although resulting in heteroploidy of an extremely large range, does not affect the basic processes of sperm formation. Hence spermatozoa are produced which vary greatly in volume, but which appear to be otherwise normal in general structure.

Material and Methods

The pentatomids concerned are *Loxa flavicollis* Drury, of which nine specimens were collected in Panama (1940 and 1941) and Costa Rica (1944); *Loxa picticornis* Horvath, represented by six specimens from Panama (1940) and Costa Rica (1944); and *Loxa florida* Van Duz, collected in Florida, U. S. A., in 1909. The data on *Loxa florida* are contained in Bowen's papers (1922a and b) although the main points here at issue are not specifically noted by him. A fourth species, *Mayrinia variegata* Dist., is represented in my material by only a single male collected in Costa Rica in 1944. Though strikingly smaller than the uniformly large species of Loxa, it is nevertheless a close relative and was formerly classified in that genus. Its affinity to the species of Loxa is now further attested by the fact that it shares with them the striking mitotic features that form the subject of this paper.

Both testes and ovaries were fixed in either Sanfelice or Allen's Bouin and stained with haematoxylin, gentian violet, or the Feulgen method. Although Bowen did not state it, it is more than likely that his material of *Loxa florida* for which he thanks Professor E. B. Wilson, was fixed in strong Flemming solution. In all of my own material the gonads were usually fixed within a short interval of capture, usually less than three hours and sometimes within a few minutes.

My thanks are due to a number of people who have aided me in collecting my material. In this respect I am especially obligated to Dr. T. J. Grant of the U. S. Department of Agriculture in Turrialba, Costa Rica.

Structure of the Testis

In all three species of Loxa, the testis is composed of seven lobes, which are arranged side by side in a single series. Following Bowen (1922b) these lobes are designated by consecutive numbers, the first being closest to the side toward which the vas deferens opens (Fig. 1). The lobes show a constant variation in diameter, but only one lobe is strikingly larger than the others. This is the fifth, which may have a diameter two or three times that of any other and which at the same time is characterized by spermatogonial and early spermatocyte cells that are distinctly smaller than the cells of other lobes. It is this lobe which regularly shows heteroploidy in the spermatocytes.

It is this lobe also which shows heteroploidy in Mayrinia. There however, the fifth is not the largest lobe, for it is exceeded in diameter by the sixth. In neither of the two Mayrinia testes at my disposal is a seventh lobe visible, and it may be that in this species the last two lobes have fused to form a single, larger one. Since there are some indications that my specimen is an old male, it is inadvisable to draw any general conclusions on this point. The main point however is fully established. In all four species it is the fifth lobe of the testis which always shows the special development here under discussion.

Cytology

In all three species of Loxa, the spermatocyte cells are smaller in the fifth than in the other lobes. But as Bowen (1922b) has pointed out in reference to polymegaly in general, this size difference rests in the volume of the cytoplasm rather than that of the chromosomes. In Mayrinia, such polymegaly is not as striking as in Loxa.

The spermatogonial divisions appear to be very much alike in all of the lobes (Fig. 2*A* and *B*). The fifth lobe shows the diploid set of 14 chromosomes differing in no perceptible way from that of the remaining lobes, and mitoses are orthodox. The unusual developments do not become evident until the prophase of the first spermatocyte. Since a detailed cytological analysis will be published elsewhere, only a brief outline will be given here.

The most obvious deviation from the normal course first occurs some time before diakinesis. When the cells of a given cyst reach this stage they begin to

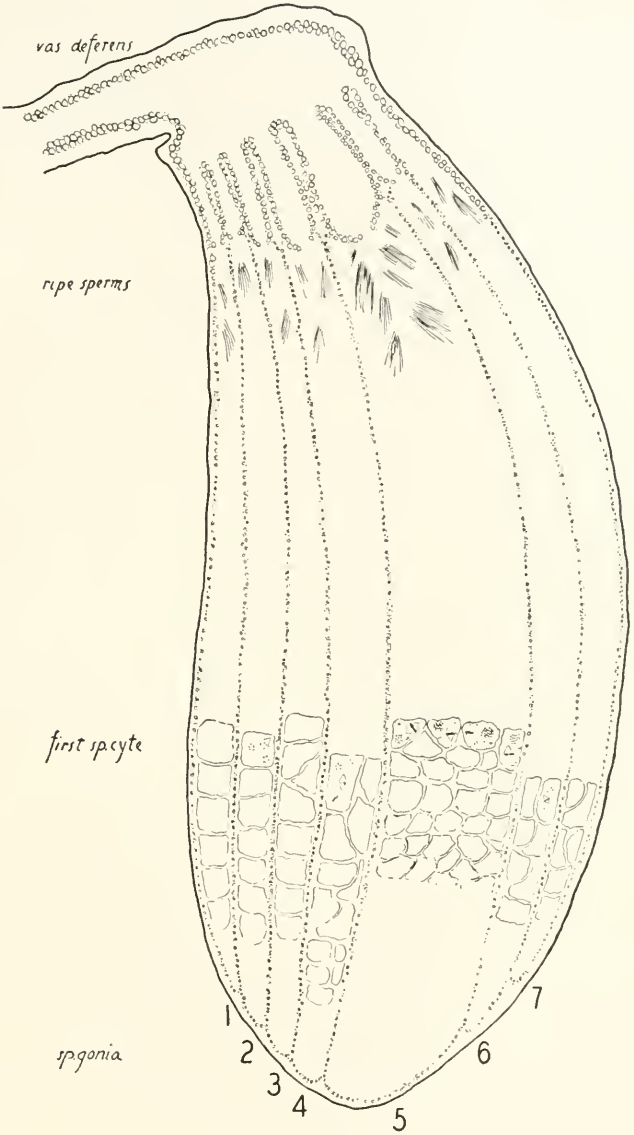

FIGURE 1. Section of the testis of *Loxa flavicollis*, showing the positions and comparative sizes of the seven lobes. Heteroploidy occurs regularly in the fifth lobe. Actual length of testis is 4 mm. and contents are only partially shown.

fuse with each other. The process is irregular in the sense that cellular aggregates resulting from the union of two or very few cells may lie side by side with much larger aggregates comprising many more cells. The nuclei at this time tend to become amoeboid and may divide by constriction, but if fusion occurs among them, it is not general.

Later during the prometaphase, cellular boundaries become very vague and the whole cyst may come to simulate a single, giant cell. It is at this time also that the nuclear walls disintegrate, as they do in the normal course of events. The chromosomes, condensing rapidly, then seem to lie scattered irregularly through the cytoplasm. However, immediately prior to the first appearance of spindles, the cytoplasm is segregated into discrete masses which do not seem to correspond exactly to the original fusion aggregates. It is probable that this new step involves the activity of both chromosomes and centrioles in the formation of spindles, and the latter appear very soon thereafter. Simultaneously the chromosomes are arranged into numerous metaphase plates, the number of which corresponds to the rounded masses of cytoplasm just formed. Since the latter seem to have no reference to the original prophase spermatocyte cells, the different metaphases may be composed of as few as two (perhaps even one) and

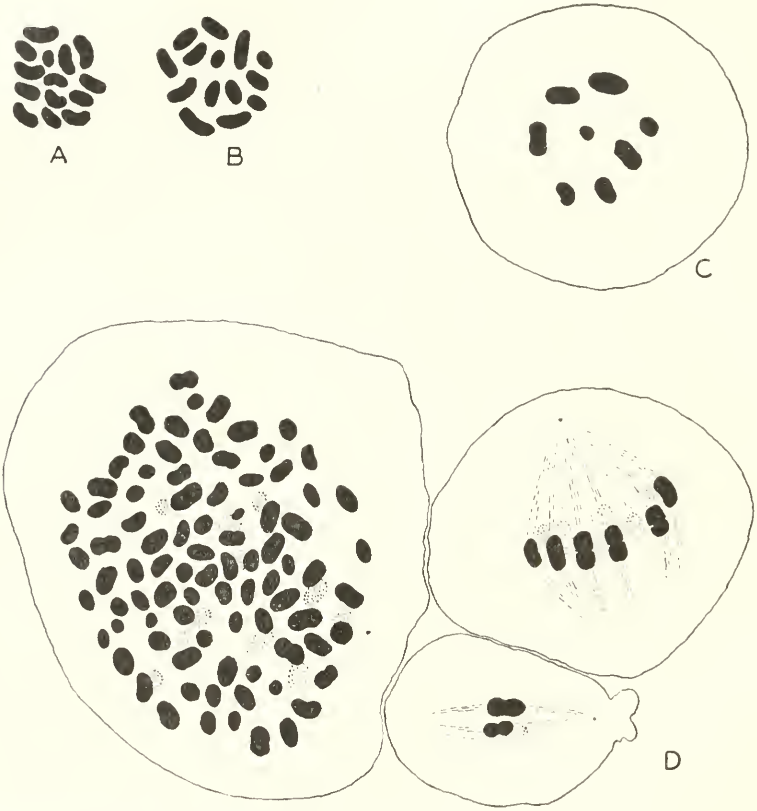

FIGURE 2. All from *Loxa flavicollis* (approx. 1,500 ×).
A. Spermatogonial metaphase from normal lobe (14 chromosomes).
B. Spermatogonial metaphase from fifth lobe (14 chromosomes).
C. First spermatocyte metaphase from normal lobe (6 autosomal tetrads + X + Y).
D. First spermatocyte metaphases from fifth lobe (showing 3, 12, and upwards of 60 chromosomal bodies).

as many as two hundred or more chromosomes (Fig. 2*D*). By the same token, the types of chromosomes included in such metaphase plates vary greatly. All in all, these configurations of chromosomes are the result of rather haphazard processes and it is probably only very rarely that they happen to represent the orthodox set of chromosomes (Fig. 2*C*) in the first spermatocyte (i.e. six autosomal tetrads + X + Y), especially since it seems doubtful whether normal pairing occurs in the fifth lobe.

Although the number of multipolar spindles is distinctly higher than in normal lobes, it is not as great as one might expect from the seemingly confused conditions that prevail just before spindle formation. Unlike the chromosomal movements in the preceding period, the maneuvers of the centrioles are evidently not completely at random and their final positions result in bipolar figures in the great majority of cases.

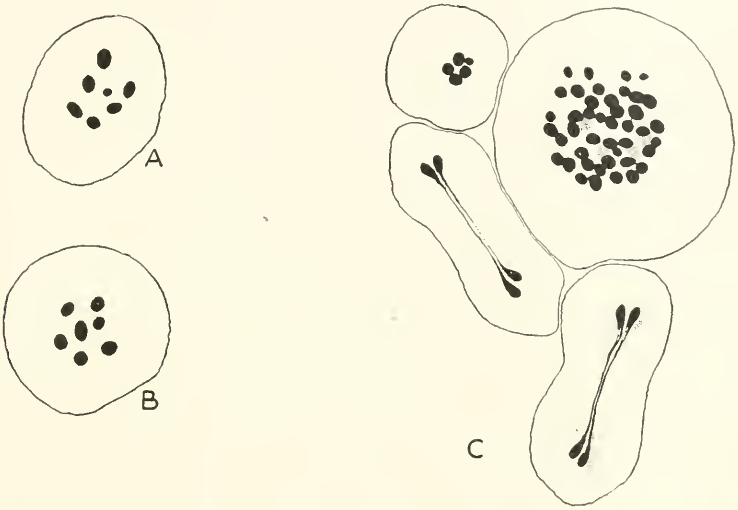

FIGURE 3. All from *Loxa flavicollis* (approx. 1,500 ×).
A. Chromosomes of telophase of second spermatocyte in normal lobe (6 autosomes + Y).
B. Chromosomes of telophase of second spermatocyte in normal lobe (6 autosomes + X).
C. Chromosomes of telophase of second spermatocyte in fifth lobe (showing 2, 4, 5, and upwards of 40 chromosomes at each pole).

Despite the bewildering range in the composition of these metaphase plates, the actual division of the abnormal first spermatocytes occurs normally. The second spermatocytes therefore carry a correspondingly great variation in the numbers of chromosomes and they too divide successfully to give spermatid cells (Fig. 3).

The nuclei that are formed in the spermatids after the second spermatocyte division of course reflect the variation in the chromosome numbers that are encountered in the meiotic divisions. Hence they may greatly exceed in size the normal spermatid nucleus, and such large nuclei may lie side by side with the tiny nuclei derived from only one or two chromosomes (Fig. 4). However except for this deviation from the normal size, the processes of sperm formation seem to parallel those observed in the normal lobes. The behavior of chondriosomes and Golgi material seems to be regular, and the transformations of the nucleus that culminate in the tenuous head of the ripe sperm are undergone just as in normal cells. The number of degenerating sperms is not great and certainly a huge number reach the final stages near the entrance to the vas deferens. Since cells with a normal complement of chromosomes must be very rare indeed, there is no escaping the fact that very many sperms carrying irregular combinations and numbers of chromosomes reach the final stages of development. Whether they then enter the egg and become functional has not been determined.

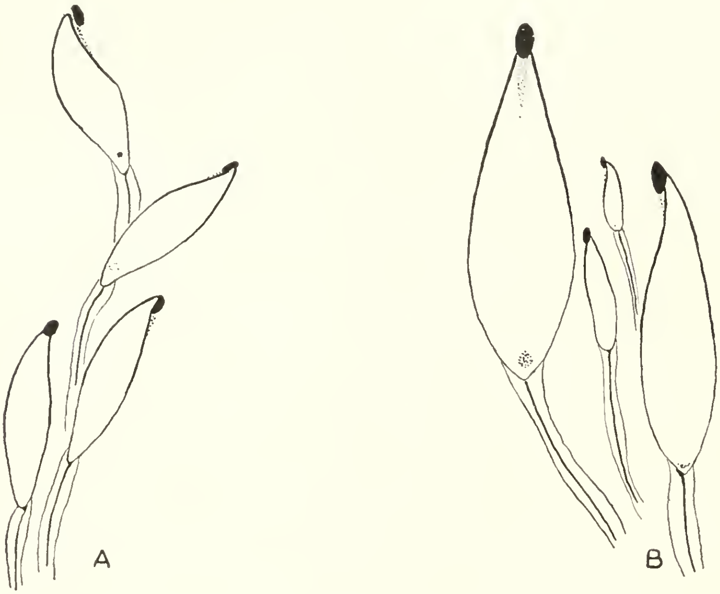

Figure 4. All from *Loxa flavicollis* (approx. 1,500 ×).
A. Sperms (only heads shown complete) at about Stage n. from normal lobe.
B. Sperms (only heads shown complete) at about Stage n. from fifth lobe, showing great variation in size.

The evidence that *Loxa florida* duplicates the conditions here described, is indirect but conclusive. In one of his papers, Bowen (1922a) discusses the occurrence of abnormally high numbers of chromosomes in that species, and ascribes them to a fusion of spermatocyte cells. He mentions that this takes place in the same lobe of both the Loxa testes available to him. Although not stating which lobe this is, Bowen characterizes it as carrying unusually small spermatogonial and early spermatocyte cells. In a somewhat later paper of that year (1922b) he mentions that in *Loxa florida* it is the fifth lobe that has cells which are distinctly smaller than those of the other lobes. There is thus no reason to doubt that he was dealing with conditions in *Loxa florida* that are essentially the same as those here described and which there occur also in the fifth lobe of the testis.

Evolutionary Aspects

These then are the astonishing conditions that confront us in the two genera, Loxa and Mayrinia. In four different species, taxonomically distinct, and collected from Panama, Costa Rica, and Florida—a latitudinal range of at least 1,700 km. (or about 1,050 miles)—one of the lobes of every testis undergoes a very special type of abnormal development. This abnormality results in a great number of unusual distributions of the chromosomes, but is in its basic nature identical in all four species. For in all of them it is always the fifth lobe of the testis that is affected; in all of them the spermatogonial divisions of this lobe seem normal and cytological indications of abnormality do not appear until the preparatory phases of the meiotic period; and in all four species the odd assortments of chromosomes that result partake nevertheless in a formation of sperms which are normal in appearance except for size.

Whatever the factors may be that bring about this constant and regularly occurring abnormality, its evolutionary aspects pose some interesting questions. It is reasonable to assume that the conditions underlying it are of long standing in the history of these species and that in fact they were probably present in the parent form from which they arose. Since all of my sixteen specimens carry the orthodox complement of 14 chromosomes, the indications are that the great majority of the sperms with abnormal chromosomes numbers are either nonfunctional or produce nonviable zygotes. The existence of so wasteful a development would seem to be an incongruity which might well be expected to meet with short shrift in the processes of natural selection. Nevertheless it has persisted, either because it involves some benefit to the species that is not immediately obvious or else because the elaboration of useless sperms, even in such huge numbers, entails only an imperceptible drain on the evolutionary welfare of the animal since it is amply compensated by the production of sperms from the normal lobes of the testis.

Be that as it may, the constant production of gametes with irregular chromosome numbers should greatly enhance the chances for producing zygotes with other than the usual complement of 14 chromosomes. Granted that most of the abnormal sperms will not produce viable zygotes, it is likely that some of the many unusual combinations do at times develop. Certainly the flow of new and heteroploid forms from such a species should be very much greater than in the case of species in which the opportunities for such varietal changes are restricted to the comparatively rare mitotic accidents that occur in the more orthodox processes of germ cell formation. It may be that a basic conservation that may exist among the Pentatomidae is not favorable to such evolutionary adventures, but the number of specimens at my disposal is surely too small to allow of any final conclusions on this point. Certain it is that a further examination of the 33 so far described species that belong to the three, taxonomically closely knit genera Loxa, Mayrinia, and Chlorocoris may well throw light on these questions (all except *Loxa florida* are exclusively neotropical, but their range extends from Florida to Argentina; see Van Duzee, 1909 and Horvath, 1925).

But if, as already suggested, the conservatism of these Hemiptera does not allow a utilization of such evolutionary opportunities, the case nevertheless presents a mechanism which in some other groups might well be conducive to rapid and great strides in the elaboration of new species. The ready viability of forms with unusual chromosome combinations such as characterizes for instance genera like Datura and Crepis would suggest that other forms might well take advantage of such a range of possibilities. At any rate, the case indicates in what unexpected ways a group of organisms may suddenly become very active in the evolutionary sense.

Differentiation of Sperms

The case reflects in a rather striking, albeit not entirely novel way, on the problem of differentiation. It is clear that the complex processes which bring about the formation of the highly specialized sperm—including the special transformations of Golgi and chondriosome material as well as the change of the spherical spermatid nucleus to the extremely tenuous, chromatic sperm head—all occur regardless of whether only one or two or many dozens of chromosomes are present in the cell. It therefore would seem safe to conclude that the immediate control over these developments is vested in cytoplasmic elements and that, if nuclear genes are primarily responsible they must exert their influence on the cytoplasm at least two mitotic cycles earlier (in the spermatocyte prophase when cell fusion first occurs). A demonstration to the same effect is contained in the work of Dobzhansky and Sturtevant (1931) where it is shown that sperms of Drosophila which have lost various sections of the chromosomes through translocation during the meiotic prophase, nevertheless become functional.

Summary

1. The fifth lobe in the testes of four species of Loxa and Mayrinia regularly produces sperms carrying heteroploid complements of chromosomes.

2. This condition must be of long evolutionary standing and may be of significance in the formation of new forms.

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ASEXUAL REPRODUCTION IN THE COLONIAL TUNICATE, BOTRYLLUS SCHLOSSERI (PALLAS) SAVIGNY, WITH SPECIAL REFERENCE TO THE DEVELOPMENTAL HISTORY OF INTERSIPHONAL BANDS OF PIGMENT CELLS

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INTRODUCTION

Botryllus schlosseri (Pallas) Savigny[2] is a colonial tunicate readily obtained in the Eel Pond at Woods Hole, Massachusetts. In a well developed colony the individual blastozooids (or ascidiozooids) are grouped into one or more systems; each system consists of 2 to 23 blastozooids radiating outwards from a central common cloaca with the separate oral siphons distributed at the periphery. The most striking feature of many Botryllus colonies is the localization of special light-reflecting pigment cells between the oral and atrial siphons of each blastozooid. Because of the association of individual blastozooids into systems within each colony the total aggregation of such reflecting cells within any one system forms an attractive star-shaped pattern (Pl. 1, Fig. 3) clearly revealing the spatial distribution of the blastozooids involved. Such patterns will be referred to as intersiphonal patterns of pigment cells (intersiphonal patterns for short), and each arm of the pattern will be called an intersiphonal band of pigment cells (intersiphonal band for short).

Such intersiphonal bands have been described more or less incidentally by various taxonomists; however, only two of these seemed to realize that the bands are actually aggregations of pigment cells in specific regions. Both of these men realized that these intersiphonal bands are not constant in their appearance. Pizon (1899a) merely states that they undergo changes with time without specifying the nature of these changes. Bancroft (1903) states that in young zooids there are no intersiphonal bands at all and that three to four days elapse before they reach their complete formation. Such brief observations constitute the only information available in the literature concerning the developmental history of intersiphonal bands

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[1] It is a pleasure to acknowledge the excellent assistance of Miss Juanita Senyard during the accumulation of the data upon which this paper is based.

[2] Many species and varieties of Botryllus have been described (Giard, 1872; Herdman, 1891; Hartmeyer, 1909–1911; Alder and Hancock, 1912). However, these were distinguished largely on the basis of color differences. Pizon (1899a) early recognized the need to revise the classification of Botryllus since he realized that considerable color variation may occur even within the same colony, and Bancroft (1903) even went so far as to state (p. 161), "*in Botryllus, as it occurs in Europe and the Atlantic Coast of North America, color characters cannot be used for separating species; . . . therefore, since none of the described species have been based upon morphological characters, there is no valid reason for recognizing more than the single species, B. schlosseri (Pallas, 1766, pp. 355–356) Savigny (1816).*" This view is acceptable to Van Name (1910) and to Herdman (1925).

of pigment cells. This lack of information is rather surprising since the bands are so very striking when fully formed (Pl. I, Fig. 5) and in view of the fact that so much has been written concerning other features of Botryllus.

One of the most interesting features of these intersiphonal bands has received no attention at all, viz., that they are not permanent additions to the pigment pattern of the colony; instead, as soon as they have formed, they are completely destroyed, and this destructive phase marks the most radical change in the appearance of the colony (Pl. II, Fig. 8). This destructive phase is inevitable since, as is well known (Berrill, 1941c), the parent zooids degenerate each time a new generation of zooids arises by asexual reproduction, and these intersiphonal bands are properties of the individual zooids, not of the colony as a whole. The fact that they give to the colony a characteristic intersiphonal pattern or group of patterns is only secondary.

This paper has a twofold purpose: (1) To describe the visible changes in the intersiphonal bands of pigment cells during the establishment of the colony from the larva. By observing the development of a number of colonies it is possible to correlate rather accurately the steps involved in the formation and destruction of the intersiphonal bands with other known steps in asexual reproduction. (2) To describe the variation in the intersiphonal patterns when different colonies are compared, and to consider possible factors involved in such variations.

MATERIALS AND METHODS

The eggs of Botryllus undergo fertilization and development up to a tadpole stage within the blastozooids. Each day some of these tadpoles escape from the cloaca. In order to obtain them adult colonies were collected from the Eel Pond in the morning and in the laboratory they were distributed in ten inch finger bowls filled with sea water. As the larvae escape from the parent zooids they swim toward the light and toward the surface (see Grave and Woodbridge, 1924, for time of liberation of larvae and for reactions of the larvae to light and gravity) and they can be collected easily with a pipette; three of them were placed into a large drop of sea water in the center of a syracuse watch glass and were left to attach and metamorphose into the oozooid. The watch glasses were stacked to prevent evaporation. Many of these tadpoles metamorphose without attaching (Grave, 1937) or exhibit certain indications of abnormal metamorphosis (Zhinkin, 1939), but these were discarded. Usually at least one tadpole attached in each watch glass; in some instances two or even all three larvae attached, and if they were sufficiently isolated from one another, they were allowed to continue their development. After attachment had occurred the watch glasses were immersed in large aquaria through which sea water was circulating. The watch glasses were inverted so debris would not settle out too thickly and obscure the development of the colonies, and they were held in this position by wooden racks. Each day these watch glasses were removed from the aquaria and the appearance of the colonies developing within them was sketched under the dissecting microscope with reflected light for illumination, since by such illumination only the distribution of the special light reflecting pigment cells is revealed and all other pigmentation of the zooids is automatically eliminated from observation (see Plates I and II). The exact time at which each sketch was made was recorded. Whenever a colony was

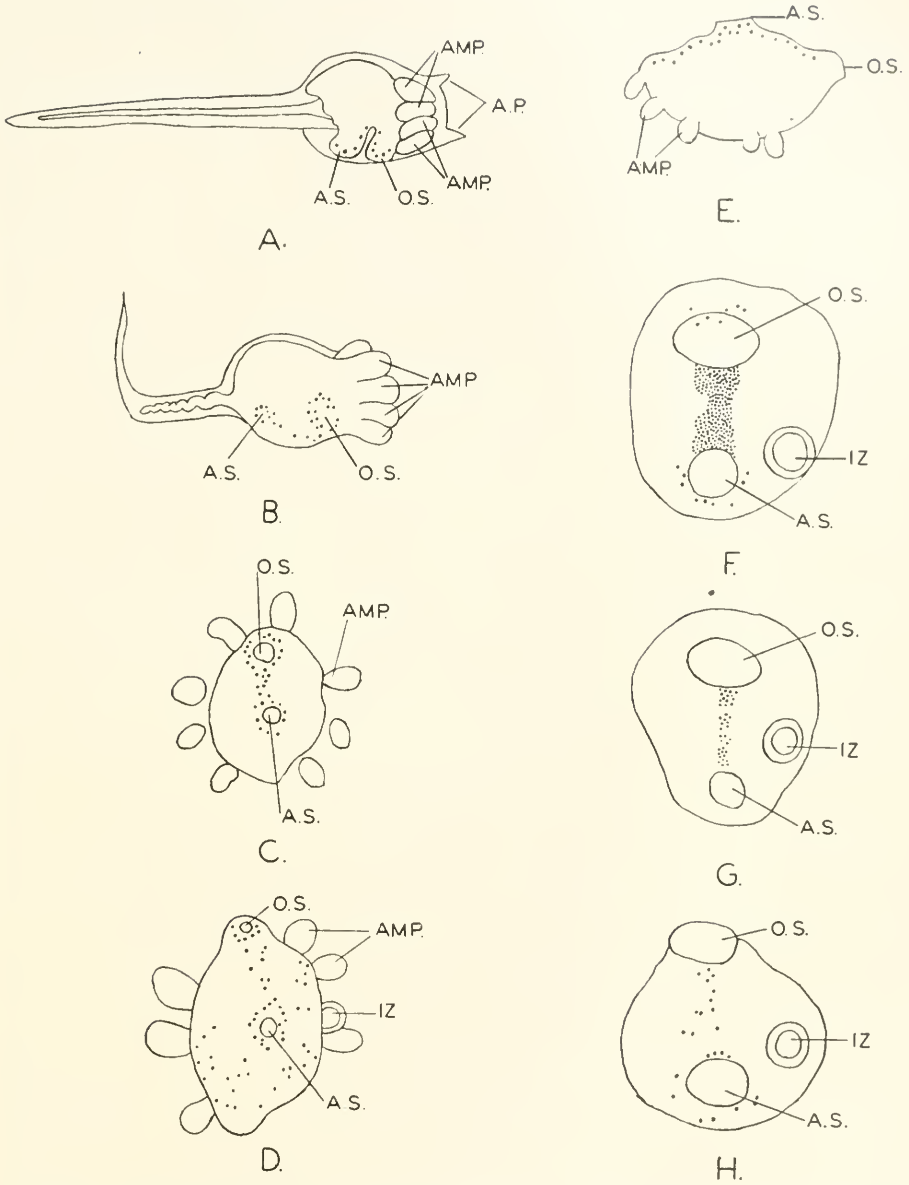


FIGURE 1. The development of intersiphonal bands of pigment cells in the oozooid. Each dot represents one pigment cell. a. Botryllus tadpole 20 to 30 minutes after escape from parent zooid. b. Tadpole in which the tail is undergoing absorption; one hour after escape from parent zooid. c. Oozooid eight hours after escape from parent zooid. d. Another oozooid 11 hours after escape from parent zooid. e. An optical section of an oozooid eight hours after escape from parent zooid. f. Oozooid No. 8c; age two days; example of a *strong* intersiphonal band of pigment cells. g. Oozooid No. 10; age two days; example of a *medium* intersiphonal band. h. Oozooid No. 18c; age two days; example of a *weak* intersiphonal band. Abbreviations: a.p. = adhesive papillae; amp. = ampullae; a.s. = atrial siphons; o.s. = oral siphons; 1Z = first generation blastozooids.

in a crucial stage of transformation it could be watched for considerable periods of time without injury. However, because of the large number of colonies studied, continuous observations on any one colony for long periods of time were not possible, although such a procedure would have yielded useful supplementary information. All sketches made for each colony were then mounted from left to right in a horizontal row on large sheets of paper in the order in which the sketches were made, and they were so arranged on these sheets that sketches of all colonies made on any one day lie in vertical columns. Consequently the changes in the appearance of any one colony throughout the period studied can be ascertained quickly by running the eye horizontally across the sheet; and similarly the appearance of all the colonies on a given day is readily compared by running the eye vertically down the sheet. Sixty-one colonies were started from isolated larvae on July 31, 1942. These were first sketched on August 2, next on August 4, and then at daily intervals until August 19 if they still survived. Due to the increased complexity of the colonies by this time it was impossible to continue daily observations on all of them. Ten colonies were therefore selected and were sketched at daily intervals until September 4. Twenty-nine other colonies were allowed to develop until August 29 when sketches were made of them; however, for these colonies the daily changes between August 19 and August 29 are unknown. In the case of the remaining 22 colonies no sketches were made after August 19 for various reasons. Consequently, since only incomplete records are available for these 22 colonies, they will not be included in the general observations given below. In order to present these observations the daily changes in the appearance of one colony (No. 38) selected as an example will be described in detail (Fig. 2); the observations on the other colonies can then be presented briefly (Table I) by merely emphasizing their similarities and differences when compared with this example. All observations have been made exclusively on living material.

OBSERVATIONS

Formation and Variation of Intersiphonal Bands in Oozooids

Since the main series of colonies started on July 31 was first examined two days later it was necessary to start other colonies (on August 31 and September 3) in order to study the establishment of the first intersiphonal band during metamorphosis of the tadpole into the oozooid. These colonies served only for these early observations on oozooid development and were then discarded. Although the tadpole is rather opaque a few reflecting pigment cells can sometimes be seen in a newly emerged larva (Fig. 1a). By transmitted light these cells appear yellowish and by reflected light they are just barely visible. They are quite definitely localized in the dorsal surface and already outline the siphonal areas. Within an hour after the escape of the larva from the parent these pigment cells have darkened

FIGURE 2. The developmental history of intersiphonal bands of pigment cells in colony No. 38. Each dot represents a pigment cell. See text for age of colony at stages illustrated and for description of the progressive changes. The various stages are not drawn accurately to scale. Each degenerating zooid is indicated by cross-hatching. Abbreviations: c.c. = common cloaca; o.s. = oral siphons; s. = space free from pigment cells; 1Z to 7Z = first to seventh generation blastozooids.

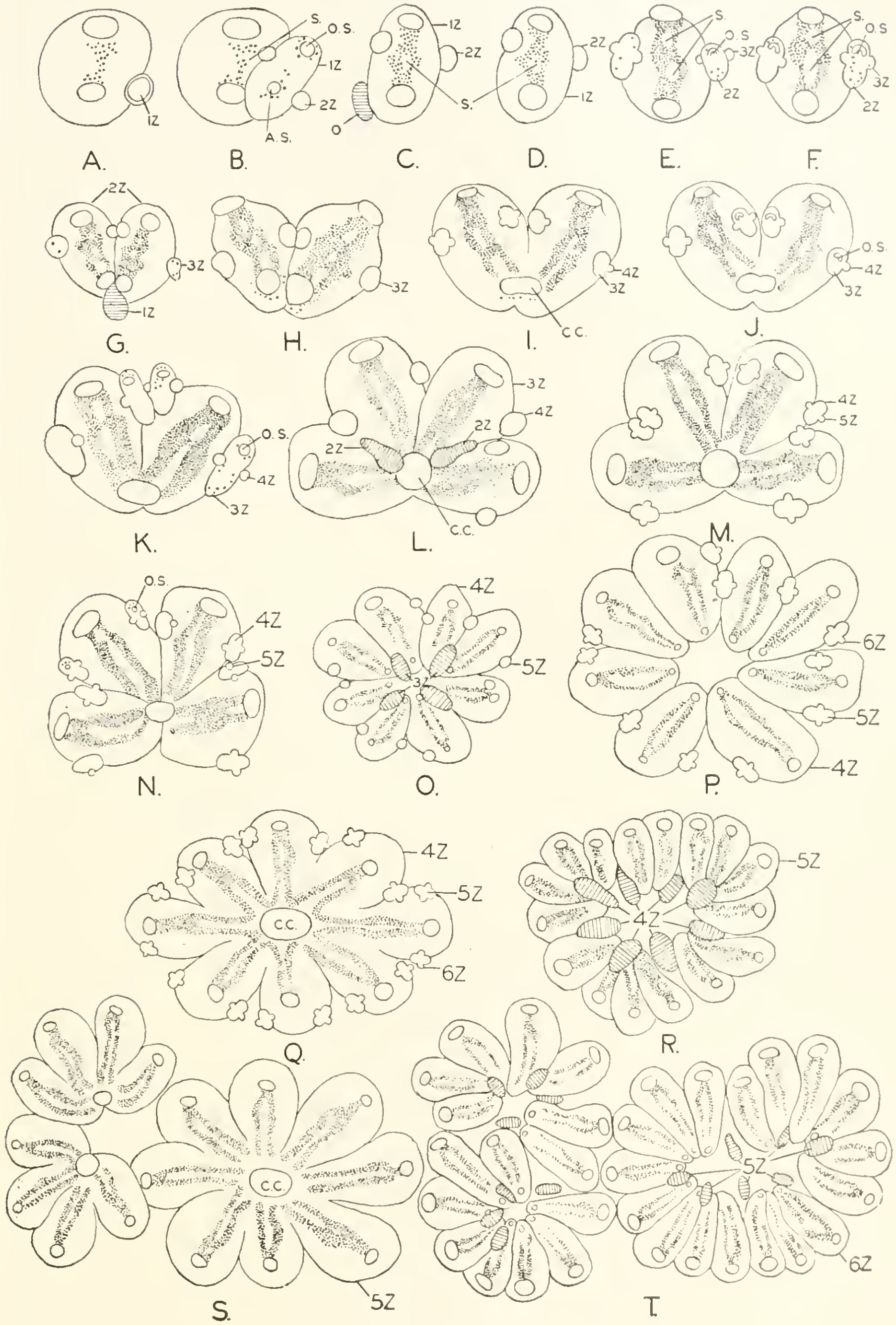

A.
1Z
B.
S.
O.S.
1Z
2Z
A.S.
C.
1Z
2Z
S.
O
D.
2Z
S.
1Z
E.
S.
O.S
3Z
2Z
F.
S.
O.S
3Z
2Z
G.
2Z
3Z
1Z
H.
3Z
I.
4Z
3Z
C.C.
J.
O.S.
4Z
3Z
K.
O.S.
4Z
3Z
L.
3Z
4Z
2Z
2Z
C.C.
M.
4Z
5Z
N.
O.S.
4Z
5Z
O.
4Z
5Z
3Z
P.
6Z
5Z
4Z
Q.
4Z
5Z
C.C.
6Z
R.
5Z
4Z
S.
C.C.
5Z
T.
5Z
6Z

FIGURE 2

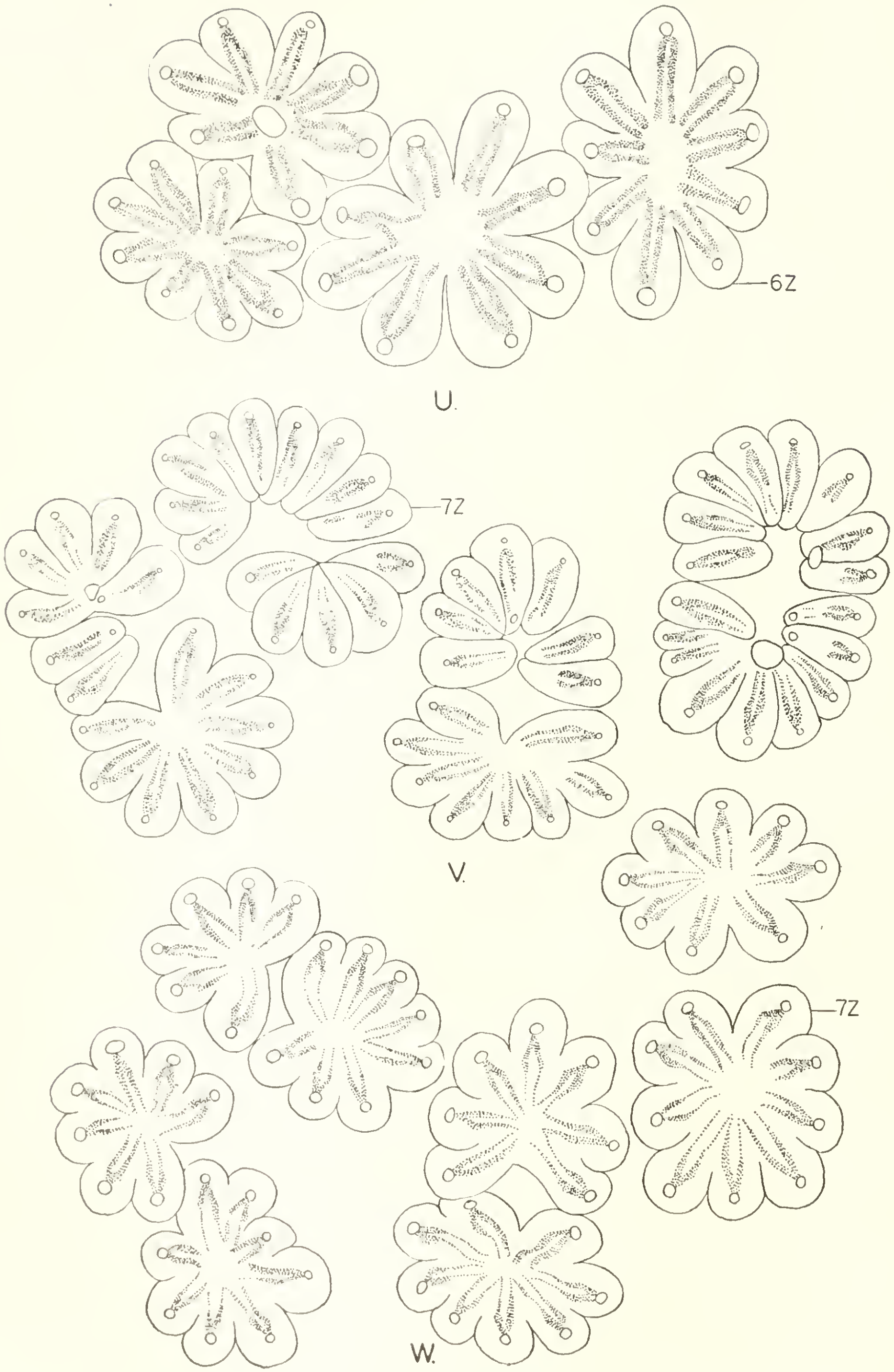


FIGURE 2—*Continued*

TABLE I

Summary of the relationship between changes in the intersiphonal bands of pigment cells and the major changes in asexual reproduction in all colonies studied. Each number indicates the percentage of colonies undergoing the change indicated. The number in parentheses following each percentage indicates the generation of zooids involved (O = oozooid, 1–7 = first 7 generations of blastozooids). The percentages indicated by the asterisk may be too small since parent zooids obscure the buds in advanced stages of colony formation.

Age in days	Zooids degenerating	Pigment cells circulating	Inter-siphonal bands single	Inter-siphonal bands double	Buds enlarging	Oral siphons present	Atrial siphons present	Common cloacas formed	Non-growing buds present
5	59.0(0)	30.7	33.3(1)	0.0(1)	59.0(2)	0.0(2)	0.0(2)	0.0(2)	0.0(3)
6	41.0(0)	33.3	61.5(1)	5.1(1)	94.9(2)	0.0(2)	0.0(2)	0.0(2)	17.9(3)
7	2.6(0)	7.7	5.1(1)	56.4(1)	100.0(2)	15.4(2)	0.0(2)	0.0(2)	94.9(3)
8	0.0(0)	7.7	7.7(1)	56.4(1)	100.0(2)	51.3(2)	28.2(2)	0.0(2)	100.0(3)
9	35.9(1)	35.9	17.9(2)	2.6(2)	15.4(3)	94.9(2)	94.9(2)	0.0(2)	0.0(4)
10	41.0(1)	23.0	28.2(2)	25.6(2)	82.1(3)	100.0(2)	100.0(2)	23.0(2)	7.7(4)
11	7.7(1)	2.6	20.5(2)	43.6(2)	97.4(3)	100.0(2)	100.0(2)	66.7(2)	74.1(4)
12	2.6(1)	12.8	7.7(2)	56.4(2)	100.0(3)	23.0(3)	0.0(3)	69.2(2)	97.4(4)
13	23.0(2)	12.8	2.6(2)	51.3(2)	5.1(4)	41.0(3)	5.1(3)	0.0(3)	97.4(4)
14	48.7(2)	38.5	30.7(3)	2.6(3)	48.7(4)	41.0(3)	41.0(3)	7.7(3)	2.6(5)
15	23.0(2)	7.7	17.9(3)	28.2(3)	84.6(4)	84.6(3)	84.6(3)	51.3(3)	20.5(5)
16	5.1(2)	2.6	7.7(3)	48.7(3)	100.0(4)	2.6(4)	0.0(4)	66.7(3)	76.9(5)
17	5.1(3)	2.6	0.0(3)	56.4(3)	100.0(4)	25.6(4)	0.0(4)	0.0(4)	89.7(5)
18	23.0(3)	7.7	5.1(4)	5.1(4)	20.5(5)	56.4(4)	15.4(4)	5.1(4)	2.6(6)
19	23.0(3)	17.9	23.0(4)	17.9(4)	87.2(5)	94.9(4)	76.9(4)	38.5(4)	10.3(6)
20	0.0(3)	0.0	10.0(4)	80.0(4)	100.0(5)	100.0(4)	90.0(4)	90.0(4)	20.6(6)
21	10.0(3)	10.0	10.0(4)	80.0(4)	100.0(5)	100.0(4)	90.0(4)	90.0(4)	80.0(6)
22	10.0(4)	10.0	0.0(5)	10.0(5)	90.0(5)	10.0(5)	0.0(5)	0.0(5)	100.0(6)
23	40.0(4)	40.0	10.0(5)	50.0(5)	60.0(6)	60.0(5)	60.0(5)	50.0(5)	0.0(7)
24	20.0(4)	20.0	10.0(5)	80.0(5)	90.0(6)	90.0(5)	90.0(5)	80.0(5)	0.0(7)
25	0.0(4)	0.0	0.0(5)	90.0(5)	100.0(6)	90.0(5)	90.0(5)	90.0(5)	0.0(7)
26	10.0(4)	20.0	0.0(5)	100.0(5)	100.0(6)	100.0(5)	100.0(5)	90.0(5)	30.0(7)
27	10.0(5)	10.0	0.0(6)	10.0(6)	100.0(6)	10.0(6)	10.0(6)	0.0(6)	*30.0(7)
28	30.0(5)	10.0	0.0(6)	40.0(6)	100.0(6)	40.0(6)	20.0(6)	20.0(6)	*40.0(7)
29	10.3(5)	12.8	2.6(6)	35.9(6)	100.0(6)	61.5(6)	61.5(6)	51.3(6)	100.0(7)
30	0.0(5)	0.0	0.0(6)	100.0(6)	100.0(7)	100.0(6)	100.0(6)	100.0(6)	0.0(8)
31	0.0(6)	0.0	0.0(6)	100.0(6)	100.0(7)	100.0(6)	100.0(6)	100.0(6)	*0.0(8)
32	0.0(6)	0.0	0.0(7)	10.0(7)	100.0(7)	10.0(7)	10.0(7)	10.0(7)	*0.0(8)
33	20.0(6)	0.0	0.0(7)	40.0(7)	100.0(7)	40.0(7)	40.0(7)	10.0(7)	*0.0(8)
34	20.0(6)	20.0	0.0(7)	80.0(7)	100.0(7)	70.0(7)	60.0(7)	60.0(7)	*0.0(8)
35	0.0(6)	0.0	0.0(7)	100.0(7)	100.0(7)	100.0(7)	100.0(7)	100.0(7)	*0.0(8)

until they appear brownish by transmitted light and are more readily visible by reflected light; they now outline the siphonal region rather sharply (Fig. 1b). In some instances one or two of these reflecting cells are also visible in the ampullae (amp., Figs. 1a–1e). No great increase in the number of reflecting cells seems to take place during metamorphosis, at least not during the first day. Seven to eight hours after emergence of the larva the siphons are readily visible and typically the reflecting cells are restricted sharply to the areas immediately surrounding and between the siphons (Fig. 1c); however, in a few instances they are more widely distributed to either side of the dorsal midline as well (Fig. 1d). That these cells are very definitely restricted to the dorsal surface of the oozooid can be seen in optical section (Fig. 1e).

When the oozooids of the colonies started July 31 were first examined on August 2 considerable variation was noted in the appearance of their intersiphonal bands; however, the intersiphonal band of any oozooid could be classified quite readily as one of three types, either: a. *strong,* in which case there is a broad, almost solid band of reflecting cells between the two siphons (Fig. 1f); b. *medium,* in which case there is a band of pigment cells between the two siphons, but it is usually rather narrow or irregular and consists of relatively few reflecting cells (Fig. 1g); c. *weak,* in which case there are only a few scattered reflecting cells near the dorsal midline (Fig. 1h) with no obvious arrangement into a band. Twenty-nine of the colonies studied arose from oozooids classified as possessing *strong* intersiphonal bands, 13 from oozooids with *medium* intersiphonal bands and 16 from those with *weak* bands. Three colonies had abnormal oozooid stages in which the intersiphonal bands could not be classified.

Destruction of Intersiphonal Bands in Oozooids and Their Formation in First Generation Blastozooids

Each oozooid reproduces asexually by budding to produce the first generation blastozooid (1Z).[3] This bud is a double walled structure located on the right side of the oozooid; its inner layer is an evagination of part of the atrial wall of the parent oozooid; its outer layer is an evagination of the overlying epidermis. The atrial portion of the bud consists of formative cells which give rise to all parts of the blastozooid except the epidermis which is derived directly from the epidermis of the parent. By the time the siphons of the first generation blastozooid are established the oozooid begins to degenerate; its intersiphonal band is destroyed and the oozooid itself almost completely disappears. Meanwhile an intersiphonal band is established in the first blastozooid.

[3] The details of asexual reproduction in Botryllus have been presented rather fully elsewhere (Della Valle, 1882; Hjort, 1892; Oka, 1892; Pizon, 1893; Berrill, 1941a, 1941b, 1941c). However, some indication of the process is necessary here and also at other points in order to understand the progressive changes in the intersiphonal patterns recorded in this paper.

PLATE I

All figures are photographs of the dorsal surface of the colonies taken by reflected light. Magnification *ca.* 7 ×.

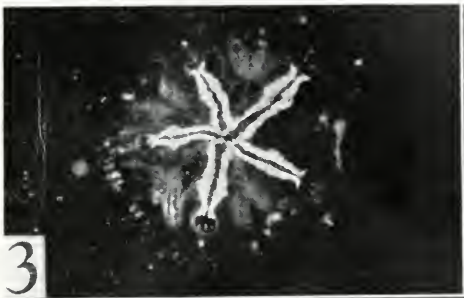
FIGURE 3. Colony 30c. Age 33 days. 6Z zooids with well developed intersiphonal bands.

FIGURE 4. Colony 35. Age 28 days. 6Z zooids distributed in two systems. Intersiphonal bands only partially developed and therefore not completely double. 5Z zooids degenerated on the 27th day.

FIGURE 5. Colony 23. Age 29 days. 6Z zooids distributed in four systems. Intersiphonal bands well developed.

FIGURE 6. Colony 13C. Age 28 days. Zooids highly sensitive and contractile resulting in the contracted state and irregularity of the intersiphonal patterns. Reflecting pigment cells are just appearing in the circulatory system. This is the first stage of the destruction of intersiphonal patterns. On the 29th day these 5Z zooids undergo degeneration and are replaced by 6Z zooids.

FIGURE 7. Colony 41. Age 28 days. 5Z zooids with well developed double intersiphonal bands. Nine 6Z zooids are faintly visible between the 5Z zooids. Note that intersiphonal bands are already appearing on 6Z zooids (arrow) before 5Z zooids have contracted. This is an exceptional condition.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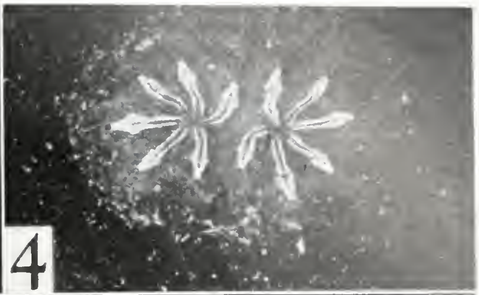
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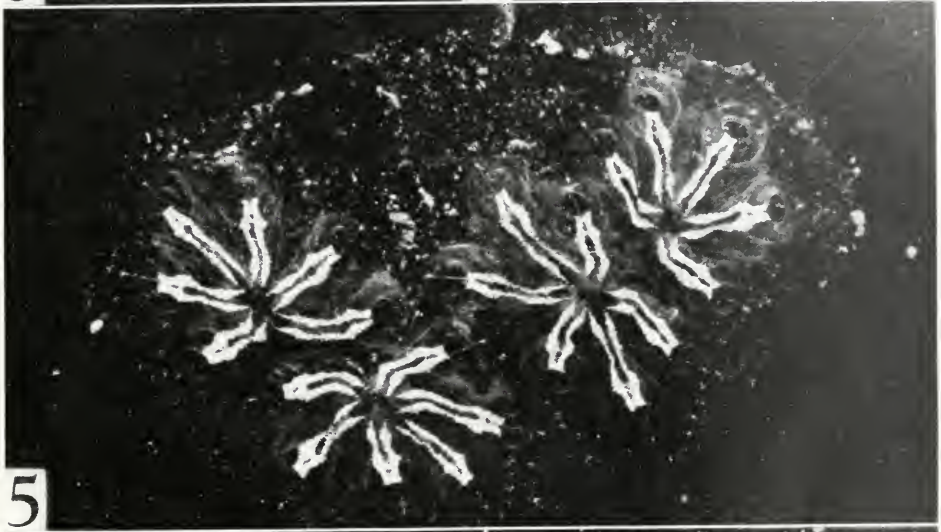
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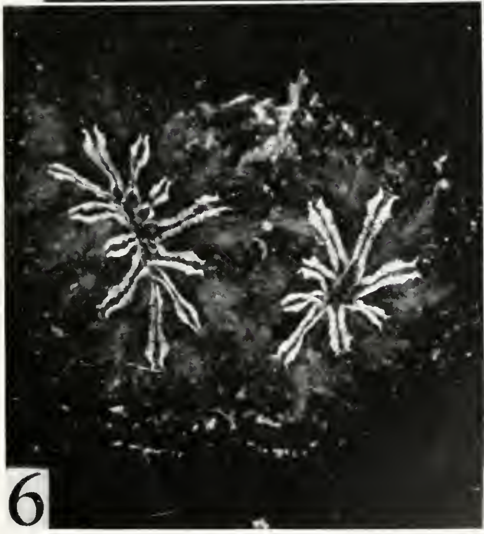
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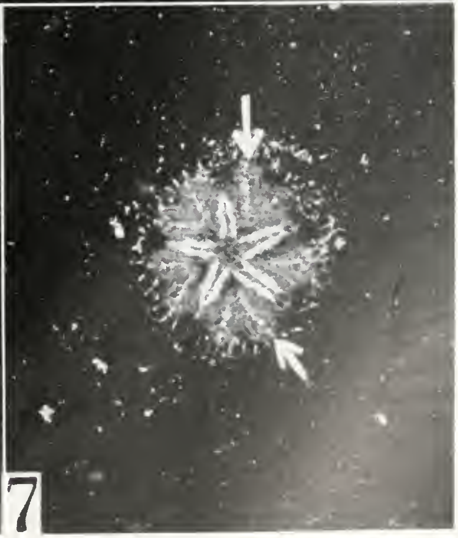
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Plate I

The asexual bud which will develop into the first generation blastozooid is already present in the tadpole before its emergence from the parent zooid (Pizon, 1893). It is readily visible on all oozooids the second day after the tadpole has attached (1Z in Figs. 1f, 1g, 1h and 2a). Sometime during the third day the bud begins to grow rapidly; a few scattered reflecting cells may then be present in the bud.

Fourth day (Fig. 2b). Both siphons are now visible in the first generation blastozooid. Reflecting cells are quite definitely restricted to the region immediately surrounding the siphons, with a few cells between the siphons and even to either side of the mid-dorsal line in some colonies. Meanwhile in the oozooid there is a tendency for the reflecting cells to become rearranged in part of the intersiphonal band, leaving spaces free from reflecting cells; such a space is forming in colony No. 38, although it is very small. In other oozooids the rearrangement of reflecting cells on the dorsal surface is much more extensive, especially near the oral siphon, serving to split that part of the originally single intersiphonal band into a double band. Occasionally reflecting cells may disappear first from other parts of the band. In three of the colonies the intersiphonal band appears somewhat contracted; in one colony some of the reflecting cells have been swept into the ampullae[4] by way of the circulatory system. The buds of the second generation blastozooids (2Z) are already present. Typically one bud forms on the right side and one on the left of the first blastozooid (although sometimes only one bud forms, in which case it is always on the right side; in other cases there may be three buds, in which case two are always on the right and one on the left).

Fifth day (Fig. 2c). The oozooid has almost completely degenerated. Usually the remnants of the oozooid are so heavily pigmented that it is impossible to identify whatever traces of the old intersiphonal band may still exist. Numerous reflecting cells arising from the disintegration of the intersiphonal band of the oozooid are now circulating freely in the blood stream and these can be traced as they move through the blood channels surrounding the siphons and in the area be-

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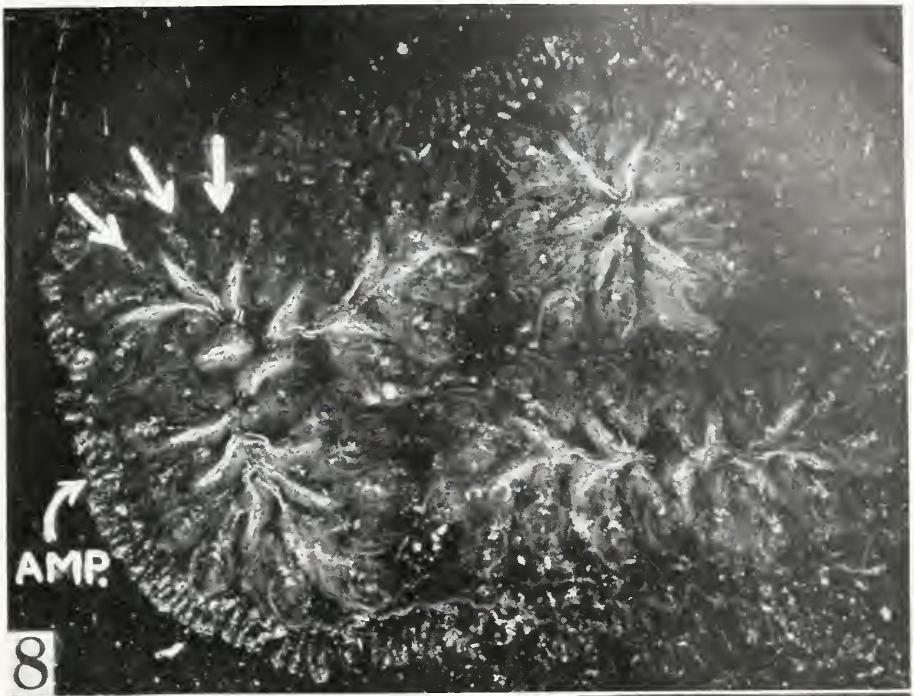
[4] Ampullae are terminal enlargements of the colonial blood vessels. Eight of them are present in the oozooid (Grave and Woodbridge, 1924); these progressively increase in number during the formation of a colony. In an adult colony they are found nearly everywhere, but are situated principally at the periphery. Several functions are attributed to them by Bancroft (1899): they act as storage reservoirs for the blood, they aid in blood propulsion since they are contractile, they act as organs for the secretion of test matrix and they probably function as respiratory structures.

PLATE II

All figures are photographs of the dorsal surface of the colonies taken by reflected light. Magnification *ca.* 7 ×.

FIGURE 8. Colony 11. Age 33 days. 6Z zooids are in maximum and permanent contraction and their intersiphonal bands are disintegrating. Reflecting pigment cells are accumulating in the ampullae at the periphery. 7Z zooids are appearing between the ampullae and the degenerating zooids and the oral extremities of their intersiphonal bands are forming (arrows). This is the second stage in the destruction of the intersiphonal patterns. On the 34th day the 7Z zooids are well formed with distinct intersiphonal bands.

FIGURE 9. Colony 35. Age 33 days. 7Z zooids are functioning and their intersiphonal bands are becoming double. Note the remains of the 6Z zooids at the center of the systems. Note also that reflecting pigment cells have almost completely disappeared from the peripheral ampullae.



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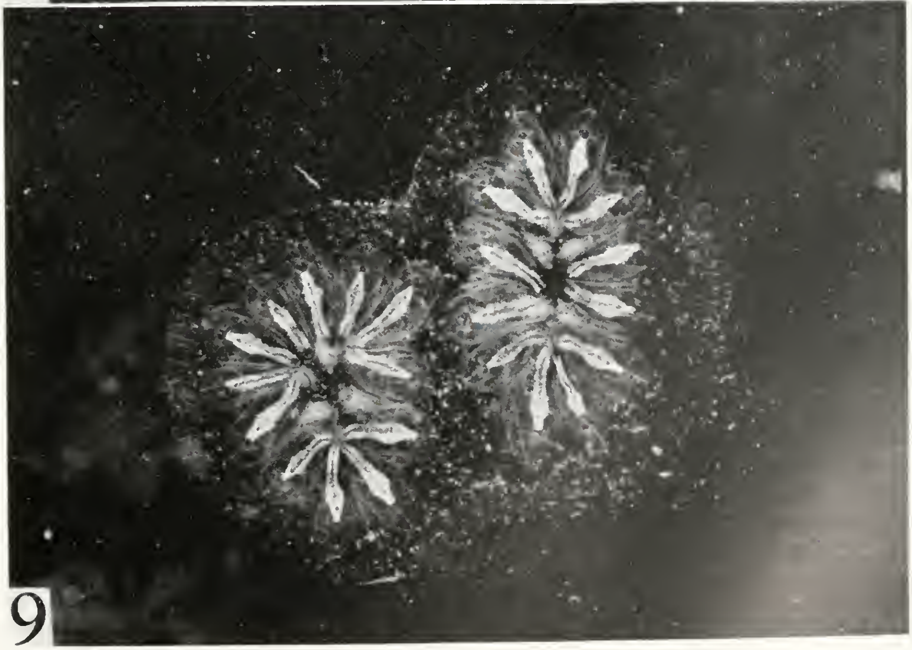

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Plate II

tween the siphons of the first blastozooid. Simultaneously the intersiphonal band of pigment cells in the first blastozooid rapidly becomes more distinct by the addition of more reflecting cells in this region. Typically a solid band of reflecting cells is established between the two siphons; this band may be almost rectangular in shape, or it may be slightly irregular. Within the band there may be one or even two spaces free from reflecting cells. Some of the reflecting cells from the degenerating intersiphonal band of the oozooid can also be followed as they are carried through the blood channels of the second generation buds. While these buds remain small the circulating pigment cells may become trapped in them temporarily which accounts for the presence of a few scattered reflecting cells early in the establishment of each generation of buds; these tend to disappear later when blood channels enlarge allowing them to move on.

In order to compare the changes in the other colonies with those described for colony No. 38 reference should be made constantly to Table I which summarizes the relationship between changes in the intersiphonal bands of pigment cells and the major changes involved in asexual reproduction in all colonies studied. For example, on the fifth day the oozooid is undergoing degeneration in 23 colonies (i.e., in 59 per cent of all colonies). In ten of these reflecting cells are contained in the vascular channels and ampullae; circulating reflecting cells are also visible in two other colonies in which the oozooids are completely destroyed. Thus pigment cells are circulating in 12 colonies (30.7 per cent). In 13 colonies (33.3 per cent) intersiphonal bands are forming in first generation blastozooids and they are all single bands (in the other 26 colonies the reflecting cells in the first generation blastozooids are too scattered to constitute a definite intersiphonal band). Second generation buds are beginning to grow in 23 colonies (59 per cent). In general the observations given below refer only to colony No. 38; in order to ascertain how characteristic these changes are for all the colonies reference must be made to Table I.

Sixth day (Fig. 2d). All traces of the oozooid have disappeared but some reflecting cells are still present in the ampullae. The intersiphonal band in the 1Z zooid is well formed; within it there is one space free from reflecting cells. In several other colonies one or two spots free from reflecting cells are now visible within the intersiphonal bands, and in two colonies this tendency for reflecting cells to disappear from the midline of the intersiphonal band is so marked that the band is now classified as double (Table I). The 2Z buds are growing, and in some colonies the 2Z buds are sufficiently developed that they in turn bear buds which will become the third generation (3Z) zooids.

Seventh day (Fig. 2e). A second space free from reflecting cells has appeared in the midst of the intersiphonal band of the 1Z zooid. Oral siphons are beginning to appear in the 2Z zooids; some scattered reflecting cells are also present in these zooids. 3Z buds are present. Reference to Table I shows that very few oozooids are actually undergoing degeneration at this time and that reflecting cells are circulating in very few colonies. Perhaps the most striking change in the appearance of the intersiphonal bands is clearly demonstrated in Table I, viz., that in many colonies the intersiphonal bands which were originally single are now definitely becoming double due to the disappearance or rearrangement of reflecting cells at one, two, three or four spots along the length of the band.

Eighth day (Fig. 2f). The intersiphonal band tends to be almost double throughout its length. Typically the disappearance of reflecting cells spreads inwards from each end of the band, so that the last part of the band to become double is the part midway between the two siphons. Oral siphons are distinctly present in 2Z buds, and in some colonies the atrial siphons have also appeared (see Table I). Reflecting cells are present in many second generation buds, but they are seldom restricted sharply to the siphonal areas.

Destruction of Intersiphonal Bands in First Generation Blastozooids and Their Formation in Second Generation Zooids; Establishment of First Intersiphonal Patterns

Ninth day (Fig. 2g). The first generation blastozooid is undergoing degeneration; reflecting cells are circulating vigorously. In all colonies where 1Z zooids are degenerating reflecting cells are circulating (Table I). The 2Z zooids have grown rapidly; they possess both oral and atrial siphons and their intersiphonal bands are well developed; three clear spots are present in the band of one zooid and two in the other. The buds which will develop into the third generation zooids are just beginning to enlarge. A few scattered reflecting cells are appearing in these 3Z buds.

Tenth day (Fig. 2h). All traces of the first generation blastozooid have disappeared; however, reflecting cells are still present in the ampullae. The degeneration of 1Z zooids is still underway in many colonies (Table I). Although the two atrial siphons of the two 2Z zooids are only closely approximated in colony No. 38 they have fused in nine colonies to form a common cloaca, thus establishing a system of zooids for the first time in the life history of these colonies. In one of these colonies consisting of three zooids two atrial siphons have fused, but the third one is still separate. In some colonies the buds which will develop into the fourth generation zooids have already made their appearance (Table I).

Eleventh day (Fig. 2i). Reflecting cells are no longer present in the circulatory system of most colonies. The intersiphonal bands of the 2Z zooids are almost completely double, especially at the atrial end of the bands. The atrial siphons of the 2Z zooids have now fused to establish a common cloaca. Therefore the individual intersiphonal bands are now united at their atrial ends for the first time to form a true intersiphonal pattern. The buds which will develop into the third generation blastozooids are enlarging; in many colonies these buds contain scattered reflecting cells with no special distribution. The buds which will become the fourth generation zooids are now present.

Twelfth day (Fig. 2j). The intersiphonal bands continue to approach the condition of completely double bands, especially at their atrial ends; consequently the intersiphonal pigment patterns tend to consist of double bands in each blastozooid (as in Pl. I, Figs. 3 and 5). In four colonies one or more of the 2Z zooids are beginning to degenerate prematurely; in two of these the 2Z zooids had first joined a common cloaca before degeneration began (in contrast to Pizon, 1893, 1899a, and 1900, who believes that one of the primary causes of premature degeneration of zooids is the failure to join a common cloaca with other zooids). Oral siphons are appearing in 3Z buds; 4Z buds are present, but are not growing.

Destruction of Intersiphonal Bands in Second Generation Blastozooids and Their Formation in Third Generation Zooids

Thirteenth day (Fig. 2k). 2Z zooids with almost completely double bands are still present, but reflecting cells are appearing in the circulatory system. The 2Z zooids are beginning to degenerate in a number of colonies; in some only one or two zooids are degenerating while others are still normal; in several the zooids are strongly contracted, but actual degeneration has not yet begun. 3Z zooids are considerably larger, oral siphons are present in them, and scattered reflecting cells are also visible, especially around the oral siphons. 4Z buds have not yet started to grow.

Fourteenth day (Fig. 2l). Second generation zooids are undergoing degeneration, as is the case in many other colonies (Table I). In two colonies only one of the second generation zooids has started to degenerate; in another colony one of the 3Z zooids is also degenerating. The 3Z zooids have their siphons well formed and the atrial siphons have already fused to form a common cloaca. The intersiphonal bands are well developed; some of them are nearly double from their earliest appearance. The 4Z buds are enlarging.

Fifteenth day (Fig. 2m). The 2Z zooids are completely absent although they are still undergoing degeneration in several other colonies. Almost no change has occurred in the appearance of the intersiphonal bands. The buds which will become the fourth generation zooids are growing rapidly and 5Z buds have now appeared.

Sixteenth-seventeenth days (Fig. 2n). The 3Z zooids form a well-defined system with almost completely double intersiphonal bands of pigment cells. One colony is striking in that the band is double in one of the two zooids, but practically non-existent in the other. Oral siphons are just appearing in 4Z buds. In two colonies some of the 3Z zooids are degenerating prematurely.

Destruction of Intersiphonal Bands in Third Generation Blastozooids and Their Formation in Fourth Generation Zooids

Eighteenth day (Fig. 2o). 3Z zooids are undergoing degeneration and reflecting cells are circulating. Intersiphonal bands are forming rapidly in 4Z zooids, and they are double from their earliest appearance. The atrial siphons of the 4Z zooids have not yet joined to form a common cloaca. 5Z buds are beginning to grow.

Nineteenth day (Fig. 2p). All traces of the 3Z zooids are now lacking. The atrial siphons of the 4Z zooids have not yet joined for cloaca formation. 5Z buds are growing rapidly and 6Z buds have appeared.

Twentieth–twenty-first days (Fig. 2q). Beyond the nineteenth day daily observations were made only on ten colonies (9c, 11, 13, 23, 30b, 35, 38, 41, 44a and 46b); therefore the observations summarized in Table I beginning with the twentieth day are based only on these ten colonies unless otherwise indicated. Colony No. 38 has well developed 4Z zooids; a distinct intersiphonal pattern is now present since the atrial siphons have joined to form a common cloaca with the almost completely double intersiphonal bands radiating outwards from it.

Destruction of Intersiphonal Bands in Fourth Generation Blastozooids and Their Formation in Fifth Generation Zooids; First Appearance of More Than One Intersiphonal Pattern in a Colony

Twenty-second day (Fig. 2r). 4Z zooids are undergoing degeneration; reflecting cells are circulating strongly. The 5Z zooids do not possess atrial siphons as yet; their intersiphonal bands are almost completely double from their first appearance. These fifth generation zooids are distributed into three groups indicative of the three systems of zooids which they will constitute later; two groups of four zooids each are separated somewhat from the remaining zooids which, though widely separated from each other, are destined to form only one system.

Twenty-third–twenty-sixth days (Fig. 2s). 5Z zooids are now well developed; all the intersiphonal bands are completely double and are radiating outwards from three common cloacas, i.e., the colony now consists of three systems of zooids and therefore of three intersiphonal patterns. 6Z buds are enlarging.

Destruction of Intersiphonal Bands in Fifth Generation Blastozooids and Their Formation in Sixth Generation Zooids

Twenty-seventh day (Fig. 2t). The 5Z zooids are undergoing degeneration and reflecting cells are circulating; all the thirty-two 6Z zooids have had double intersiphonal bands almost from their earliest appearance. These zooids are distributed in four groups indicative of the four systems they will establish, although no common cloacas have yet formed; zooids are distributed in groups of seven, eight, eight and nine.

Twenty-eighth–thirty-first days (Fig. 2u). Four well-defined systems of 6Z zooids are now present, each with a common cloaca and an intersiphonal pattern. The observations recorded in Table I for the twenty-ninth day are based on all 39 colonies, i.e., on the 10 colonies on which daily observations have been made, as well as on the 29 colonies which have not been examined since August 19. The observations recorded beyond the twenty-ninth day are again based only on ten colonies.

Destruction of Intersiphonal Bands in Sixth Generation Blastozooids and Their Formation in Seventh Generation Zooids

Thirty-second day (Fig. 2v). Colony No. 38 has changed from the sixth to the seventh generation of zooids; all of the new intersiphonal bands are almost completely double; the zooids are already arranging themselves into eight groups, but only a few atrial siphons have fused as yet. There is a total of 61 zooids.

Thirty-third–thirty-fifth days (Fig. 2w). The 7Z zooids are now arranged in eight distinct systems, each with its distinct intersiphonal pattern consisting of double intersiphonal bands.

Discussion

Literature

Attention has frequently been called to the brilliant and varied coloring of the flower-like groups of ascidiozooids which constitute Botryllus colonies (M'Intosh, 1901; Herdman, 1925). According to Webb (1939), "It appears that all brightly coloured species owe their colour to pigment cells which are morphologically blood cells, though in most cases they do not participate much in the general circulation but become more or less permanently lodged along the walls of the peripheral vessels" (p. 505). For the most part, however, only incidental observations have been recorded concerning intersiphonal bands of pigment cells. Pallas (1774) illustrates several Botryllus systems in his Plate IV, Figs. 2, 3, 4 and 5, and double intersiphonal bands are clearly indicated. Krohn (1869b) in his explanation of his Plate XIV, Fig. 2 calls attention to "Breiter kreideweisser Pigmentstreifen zwischen den beiden Leibesöffnungen." Van Name (1910) states that "During life, . . . , the zooids, especially their anterior ends and dorsal portions, and the bulbs of the test vessels, are marked with a light-colored pigment, which mostly disappears after death" (p. 352). In more detailed descriptions of Botryllus colonies the presence of some sort of intersiphonal bands has been noted in numerous instances. This is especially true of *Botryllus violaceus* (Milne-Edwards, 1842). In this species (?) Giard (1872) notes (p. 621), "Les deux orifices sont reliés entre eux par des lignes plus ou moins larges, d'une coloration qui varie entre le blanc pur et le jaune brunâtre en passant par toutes les teintes intermédiaires." Concerning this same species (?) Alder and Hancock (1912) state (p. 70), "the thorax from near that [oral] aperture downwards [is] marked with a double line of opaque white, broadest above and ending on the margin of the tubular common orifice; these lines [are] sometimes yellowish and occasionally broader and confined more exclusively to the base." In *Botryllus smaragdus* Giard (1872) mentions the presence of "Lignes radiales d'un jaune plus ou moins vif, parfois à peine indiquées" (p. 626) and Pizon (1899a) describes "des lignes radiales jaunes qui s'étendent, avec une largeur et des nuances très variables, sur la face supérieure de la branchie" (p. 396). Similar radial lines extending from the oral to the atrial siphons have been described by Giard (1872) in *Botryllus aurolineatus* and *Botryllus morio,* by Alder and Hancock (1912) in *Botryllus miniatus* and by Van Name (1931) in *Botryllus primigenus.* Of these authors only Pizon definitely states that the pigment granules constituting the intersiphonal bands are contained within special cells called chromatocytes, and that these granules are about 1 μ in size and are animated by Brownian movement. Van Name (1902) also notes that in the closely related *Botrylloides nigrum* similar white pigment is contained in opaque oval cells of the same size as those containing the ground color.

The only extensive observations concerning intersiphonal bands in Botryllus are recorded by Bancroft (1903). He refers to these bands in one of two ways: as dorsal double bands, or as two parallel white bands extending from cloaca to branchial orifice; he also speaks of the blastozooids possessing such bands as white-striped zooids. Although he does not state so specifically he realizes that the pigment involved is contained in specific pigment cells. He believes that the position of these bands does not depend on that of blood vessels. His paper deals to a considerable extent with color variations in Botryllus. His comments concerning variations in intersiphonal bands will be incorporated into the discussion below.

Repeated Destruction and Formation of Intersiphonal Bands of Pigment Cells During the Development of a Colony

The intersiphonal band which develops in the oozooid is usually a solid band of cells between oral and atrial siphons. This band arises rather rapidly during metamorphosis of the tadpole and undergoes few changes while the oozooid persists, except that it sometimes tends to become double at its oral extremity. This transformation of single intersiphonal bands into double bands occurs quite slowly in the oozooids and is rarely completed before the oozooid degenerates, so that single or at best partially double intersiphonal bands are characteristic of oozooids at their highest state of development. By the third day after attachment the first generation blastozooid is growing rapidly and scattered reflecting cells are present in it. By the fourth day both of its siphons are indicated and its reflecting cells are quite definitely restricted to the region immediately surrounding the siphons, with a few scattered cells between the siphons. The oozooid then begins to degenerate and the intersiphonal band of the oozooid is destroyed. The pigment cells set free from the intersiphonal band are then present in the circulatory channels and are carried passively by the blood stream. Such degenerative changes occur in the oozooids of the majority of colonies on the fifth day (Table I). Simultaneously intersiphonal bands are forming in the first generation blastozooids, and they are all single bands. 2Z zooids are just beginning to grow rapidly, but they possess neither oral nor atrial siphons as yet. 3Z buds are not present. On the sixth day fewer oozooids are degenerating; most of the colonies have single intersiphonal bands forming in 1Z zooids, but in a few colonies these bands are transforming into double bands. 2Z zooids are growing rapidly, although they still possess neither oral nor atrial siphons. 3Z buds are now appearing. By the seventh day degeneration of oozooids is nearly completed and reflecting cells are circulating in few colonies. The most striking change has been the transformation of almost all single intersiphonal bands into double bands. Oral siphons are just appearing in 2Z zooids and 3Z buds are now present in almost all colonies. By the eighth day the first generation blastozooids are in their optimal state of development. The characteristics of this highly developed condition are as follows: (a) almost no reflecting cells are circulating, indicating that oozooids have all degenerated some time ago and that first generation blastozooids have not yet started to degenerate; (b) the intersiphonal bands of first generation blastozooids are almost all double, a condition which is usually realized only when the zooids are fully developed; (c) all 2Z zooids are growing rapidly and both oral and atrial siphons are forming in them; (d) 3Z buds are present in all colonies.

Then, on the ninth day the 1Z zooids begin to degenerate, accompanied by the appearance of reflecting cells in the circulatory system once more, and accompanied also by the onset of rapid growth of 3Z zooids. Degeneration of first generation zooids and their intersiphonal bands is at a maximum on the tenth day. Intersiphonal bands are forming in 2Z zooids, and note especially that there are almost as many colonies with double bands as with single bands at this time. For the first time the atrial siphons of 2Z zooids are fusing in some colonies to establish a common cloaca and thus to establish a system of zooids within each colony, thereby allowing establishment of the first intersiphonal patterns. 4Z buds are beginning to appear. By the twelfth day the 2Z zooids have attained their maximum development. The characteristics of this highly developed condition of the colonies are the same as those when 1Z zooids were best developed, viz., few reflecting cells are circulating, almost all intersiphonal bands are double, all 3Z zooids are growing rapidly, and 4Z zooids are present in almost all colonies. In addition atrial siphons of 2Z zooids have fused to form common cloacas in most colonies.

Degeneration of 2Z zooids begins on the thirteenth day, reaching its maximum on the fourteenth day. Most of the intersiphonal bands forming in 3Z zooids are single at this time. The atrial siphons of 3Z zooids are beginning to fuse to establish common cloacas. 3Z zooids reach their maximum development on the seventeenth day. The new intersiphonal pattern characteristic of the colony at this time is more complex than previously since it consists of more intersiphonal bands. Again the characteristics of the colonies at this moment of maximum development are the same as at the time of maximum development of 1Z and 2Z zooids.

3Z zooids then begin to degenerate and 4Z zooids attain their maximum development on the twenty-first day. Then the 4Z zooids degenerate and 5Z zooids reach their maximum development on the twenty-sixth day. These in turn degenerate and 6Z zooids attain maximum development on the thirty-first day. The 6Z zooids then degenerate and 7Z zooids are in their most highly developed condition on the thirty-fifth day. 5Z, 6Z and 7Z zooids are characterized by double intersiphonal bands almost from their earliest appearance. Thus under laboratory conditions a period of 4 to 5 days elapses between the highest state of development of one generation of zooids (and its intersiphonal bands) and the highest state of development of the next generation (and its intersiphonal bands).[5]

During each brief period of four to five days the intersiphonal bands undergo an orderly series of stepwise transformations. a. The pigment cells which constitute a new intersiphonal band first appear near the oral siphons. They are fairly scattered at first, but very soon they form a solid band of cells in the dorsal midline extending from oral to atrial siphons. b. The originally solid bands of reflecting cells then begin to separate lengthwise so that each intersiphonal band shows indications of becoming double throughout its length (Pl. II, Fig. 9). These bands progressively become more completely double (Pl. I, Fig. 4) until they are distinctly double (Pl. I, Fig. 3). The pigment cells then reflect light to the maximal extent and the particular generation of zooids involved has then attained its highest state of development. Bancroft (1903) also recognized that these intersiphonal bands are progressively built up. Pizon (1899a) realized that the intersiphonal bands change with time, but he gave no description of the changes. c. The most radical change in the intersiphonal patterns then occurs. They assume a very irregular appearance (Pl. I, Fig. 6) as compared with their extremely regular appearance at the time of their greatest development (Pl. I, Figs. 3 and 5). This irregularity is due to the contraction of some of the intersiphonal bands, due in turn to the contraction of the zooids involved. At a given instant some intersiphonal bands are almost completely contracted, others only partially, but an instant later different bands become contracted and those formerly contracted are then somewhat relaxed, with the result that the intersiphonal patterns are rapidly changing their configuration. The intersiphonal bands then undergo maximum and permanent contraction (Pl. II, Fig. 8), and at this time the intersiphonal bands are destroyed, i.e., the reflecting pigment cells constituting them are released from the band and pass into the blood channels between and around the siphons and degeneration of the zooids is underway. These circulating pigment cells are swept quickly into the ampullae (amp., Pl. II, Fig. 8) at the periphery of the colony and into the blood channels of the next generation blastozooids and can be traced as they circulate around and between the siphons of these new zooids. These reflecting pigment cells sometimes begin to circulate even before the atrial siphons have appeared in the new generation zooids, and they are usually circulating strongly when the degenerating zooids are still of adult size, are three-fourths that size, are one-half as large as adults, and even when they are completely reduced.

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[5] This period of four to five days between the maximum development of successive generations of zooids agrees with the intervals established by Berrill (1935a). However, it should be emphasized, as Bancroft (1903) and Grave (1933) have already done, that growth of Botryllus is less vigorous in aquaria than in its natural habitat. In colonies growing in the Eel Pond, Grave observed that the interval between successive generations is reduced to 2 or 3 days.

While the old intersiphonal bands are undergoing destruction new intersiphonal bands are forming in the new generation zooids (Pl. II, Fig. 8, arrows). Because of the almost perfect coincidence in the time of destruction of the intersiphonal bands in one generation of zooids and their establishment in the next generation it seemed certain that some of the circulating reflecting cells must become secondarily attached in the intersiphonal region of the next generation of zooids to participate in the formation of its intersiphonal bands. This seemed all the more certain in view of the fact that during the final rapid development of these new bands of pigment cells the reflecting cells disappear almost completely from the circulatory system (compare peripheral regions of Pl. II, Figs. 8 and 9). However, in some exceptional cases (Pl. I, Fig. 7) it is evident that new intersiphonal bands (arrows) can originate even before contraction and subsequent destruction of the bands of the old generation zooids has begun. This suggests that the first reflecting cells to appear in the intersiphonal region of the new zooids arise *in situ*. Whether all the pigment cells of the new bands arise locally, or whether they are augmented with cells from the intersiphonal bands of the degenerating zooids can be determined only by experimentally preventing the reflecting cells from the degenerating pigment bands from entering the new generation zooids, as well as by a careful histological study. Bancroft (1903) has recorded an observation of interest in this connection. In his studies on the fusion of Botryllus colonies he has observed that a single system may contain zooids derived from two different colonies; the zooids from one colony may possess well developed double intersiphonal bands whereas those of the other may possess no visible bands (Pl. XVII, Fig. 23). In his discussion (pp. 174–175) he states that "In the fused colonies the zooids may differ in color; and not the slightest tendency toward an equalization of this difference could be detected." This evidence would suggest that intersiphonal bands arise *in situ* or not at all; it suggests that even if reflecting pigment cells are present in part of a colony they can never participate in the formation of a pigment band in zooids which do not inherently possess one. If the reflecting cells from the degenerating pigment bands are not involved in the establishment of new intersiphonal patterns, perhaps they too are used as food material by the newly forming zooids; it is known that all other cells arising from degeneration of the zooids are so utilized.

Eventually the number of zooids in a colony has increased to the point where they can no longer gather around a single common cloaca. More than a single common cloaca then forms, and some zooids assemble around each with the result that the colony then consists of more than one system of zooids. Since the intersiphonal bands of one system constitute one intersiphonal pattern, the colony then has more than one intersiphonal pattern. As more and more generations of zooids develop the number of systems, and therefore the number of intersiphonal patterns, progressively increases. Krohn (1869a and b) clearly recognized that as each new generation of buds matures, the old systems are constantly replaced by new ones which usually correspond with the parent system neither in their form nor in the number of single individuals composing them.

Thus it is evident that any Botryllus colony is characterized by a specific intersiphonal pattern or group of patterns for only a very brief period. Any given intersiphonal pattern is then rapidly destroyed within a few hours and an entirely different pattern or group of patterns is built up, only to be destroyed in turn. Moreover, it is evident that this progressive remodeling of the intersiphonal patterns within a given colony is intimately associated with the process of asexual reproduction, so intimately in fact as to verify the statement by Berrill (1941c) to the effect that there is a "rigorous correlation of the features characterizing any given stage, so that if the developmental stage of one feature is known the stage as a whole can be accurately defined" (p. 100).

Variations in Intersiphonal Patterns When Different Colonies Are Compared

Some Colonies Lack Distinct Intersiphonal Patterns

Some colonies are collected in nature in which the intersiphonal patterns are not strongly expressed even when the zooids are in their maximum state of development. It is interesting to inquire whether this weakness in expression of the pattern in certain colonies is merely due to lack of proper environmental conditions, whether it is due to an unhealthy condition of the colony, whether it is a temporary condition characteristic only of that particular generation of zooids which is functioning at that instant, or whether it is a permanent characteristic of that particular colony appearing in all generations of zooids. If such a weakness of expression is a constant characteristic in certain colonies, perhaps it is genetically determined.

Two days after attachment of the larvae considerable variation is evident when the intersiphonal bands of different oozooids are compared. Some oozooids exhibit a *strong* intersiphonal band, i.e., there is a broad, almost solid band of reflecting cells between the two siphons; others possess a *medium* band, i.e., there is a rather narrow or irregular band consisting of relatively few reflecting cells; still others possess a *weak* intersiphonal band, i.e., there are only a few reflecting cells near the dorsal midline with no obvious arrangement into a band. In 15 of the colonies on the sixth day the reflecting cells are not concentrated into a distinct intersiphonal band in 1Z zooids. Of these, 11 were derived from oozooids originally classified as possessing *weak* bands and 4 from oozooids with *medium* bands; all of the oozooids whose intersiphonal bands were originally classified as *weak* (and not omitted from the observations recorded in this paper) are among these 15 colonies, but 6 colonies derived from oozooids whose bands were originally classified as *medium* are not in this group. No blastozooids derived from oozooids which exhibited *strong* intersiphonal bands are found in this group. On the eighth day 14 of the 15 colonies still do not have the reflecting cells arranged into a definite intersiphonal band, and no distinct intersiphonal band is formed before the 1Z zooids degenerate. By the eleventh day 25 colonies possess well developed intersiphonal bands in 2Z zooids, but such bands are lacking in 14 colonies. All of the colonies derived from oozooids originally possessing *weak* bands are among these 14, as well as 3 from oozooids whose intersiphonal bands were originally classified as *medium;* not a single colony derived from an oozooid exhibiting *strong* intersiphonal bands is to be found in this group. Moreover no distinct bands appear in these 14 colonies before the 2Z zooids degenerate. On the sixteenth day, although distinct bands are present in 4Z zooids in 22 colonies, no such bands exist in 15 colonies, and all colonies originally exhibiting *weak* bands are included among these 15. On the twenty-ninth day three colonies originating from oozooids with *weak* intersiphonal bands possess no distinct intersiphonal bands in 5Z zooids; two colonies are undergoing the transformation from fifth to sixth generations and therefore the expression of the intersiphonal bands could not be determined; six possess no distinct bands in 6Z zooids. Two other 5Z colonies and three 6Z colonies are now classified as possessing no distinct intersiphonal patterns; these were derived from oozooids originally classified as possessing *medium* intersiphonal bands.

Thus all colonies in which only *weak* intersiphonal bands form in the oozooid stage form only *weak* bands throughout their development, at least through the seventh generation of blastozooids. This weakness of expression of the intersiphonal bands is therefore a constant characteristic throughout the developmental history of certain colonies. On the contrary, oozooids which possess well developed (*strong*) intersiphonal bands develop into colonies which always have well developed bands. This difference between *weak* and *strong* expression of intersiphonal bands is therefore a constant one and is probably genetically determined. In the present study no attempt was made to determine whether the tadpoles which give rise to oozooids with *weak* intersiphonal bands are derived only from adult colonies in which no distinct bands occur, or whether they can come from adults possessing distinct bands. However, Bancroft (1903) has recorded some observations of interest in this connection. He collected tadpoles from three mother colonies. The tadpoles from the first mother colony gave rise to a large number of colonies called Family I; tadpoles from the second mother colony established Family II; and Family III originated from tadpoles which emerged from the third mother colony. He observed that double bands formed only in Family II and Family III, and the mother colonies of both these families had well developed dorsal bands. He also noted that in Family II two colonies developed dorsal double bands, whereas two had no trace of them. This may indicate that colonies with *strong* intersiphonal bands can be established only from tadpoles which emerge from mother colonies with well developed bands; however, colonies with *weak* intersiphonal bands can also come from tadpoles emerging from colonies with well developed bands. Should there prove to be a genetic basis for this difference between *strong* and *weak* expression of intersiphonal bands, it appears likely that the *strong* expression will prove to be dominant.

The category of *medium* as applied to the classification of intersiphonal bands appears to be an artificial one, since colonies derived from oozooids classified as *medium* are subsequently classified in part with the group lacking distinct bands in later generations, but mostly with the group possessing such bands. Thus the group of oozooids classified as *medium* is probably a heterogeneous group, part of its members actually belonging among the *weak* group, but showing the maximum development of the bands to be found in this group, and the rest belonging to the *strong* group, but showing only a minimal development of the bands. The classification of *medium* is therefore discarded as of no significance.

The Number of Zooids in a Given Generation Is Not Constant

Due to differences in the number of buds per zooid

Usually throughout the developmental history of any colony the number of zooids progressively increases and accordingly as each new generation of zooids becomes mature, the intersiphonal pattern of the colony becomes more complex than when the previous generation of zooids was present. However, the complexity of the pattern is not the same in all colonies even when they are in the same stage of development. This is evident if the intersiphonal patterns of three of the colonies illustrated in Plate I are compared. These 3 colonies are all in the same stage of development, i.e., sixth generation zooids are functioning in each, but in the colony illustrated in Figure 3 there are only 5 zooids in the 6Z generation, in the colony illustrated in Figure 4 there are twelve 6Z zooids, while in the colony illustrated by Figure 5 there are twenty-three 6Z zooids. Obviously even when colonies of the same stage of development are compared variations in their intersiphonal patterns still exist due to differences in the number of zooids, which is due mainly to differences in the rate of budding in different colonies. A question then arises concerning the factors which determine the number of buds produced by any zooid. Is the number of buds produced by a single zooid purely a fortuitous response to the environment, varying from generation to generation within any given colony? Or, is the number of buds produced by each zooid constant from generation to generation in a given colony and variable only when different colonies are compared? Any regularity in budding within one colony may possibly indicate that the budding pattern is a genetic characteristic of a colony.

According to Pizon (1893) both lateral walls of the atrium of the tadpole undergo a localized thickening sometime before the tadpole escapes from the parent. Such a thickening is the first step in the development of an asexual bud. Thus budding in the oozooid begins bilaterally, but the thickening on the left is always much less accentuated than the one on the right. By the time the tadpole has escaped from the parent the bud on the left has entirely disappeared, whereas the one on the right has developed sufficiently that the beginnings of a pharynx and two atrial sacs are recognizable. He notes that the intestine in the oozooid is crowded to the left and he believes that the pressure exerted by the intestine against the atrial wall most likely inhibits the morphogenesis of the left bud at an early stage. Most ascidiologists agree that the oozooid gives rise to a single bud on its right side, although Giard (1872) and Jourdain (1886) believe that the oozooid generally produces two first generation blastozooids.

According to Herdman (1925) bilateral budding is then the rule in the blastozooids, so that theoretically the colony should consist of a number of zooids represented by some power of two. Pizon (1899a and 1900) also regards as normal the production of two buds, one on the right and one on the left of the parent, although in many instances only one bud develops, and in these cases he states that the single bud may be either on the right or left side (1893). However, Berrill (1941b) has noted that there is usually a size difference between bud primordia on the right and left sides, the one on the right being the larger. Moreover, Oka (1892) states that whenever an equal number of buds is developing on the right and left sides of the parent those on the left are always somewhat retarded. It is therefore not surprising that Oka finds that whenever a single bud is produced, it is always on the right side. Whenever one bud is lacking Pizon always finds a feeble diverticulum of the atrial wall where that bud should have developed, indicating that budding of the blastozooid is always bilateral initially regardless of whether both buds actually continue to develop. Krohn (1869a) and Della Valle (1882) on the contrary regard the production of a single bud as the rule among blastozooids and the production of more than one bud per zooid as the exception. However, Krohn recognizes that two buds can form, in which case one is always on the right and the other on the left.

Oka (1892) has also observed that many times the original atrial thickening is not entirely used up in the production of one bud leaving a portion of the budding zone unused. Sometimes this remnant of budding material gives rise to a second bud on that same side of the parent; such a second bud is always younger in its developmental stage than the first bud developed on that side of the body. Della Valle (1882) also recognized that when more than one bud develops on the same side of the body the first one to appear is always in advance of the second in its stage of development. Pizon (1893) has observed one young colony of Botryllus in which each blastozooid carried two buds on each side; he states that the same peribranchial thickening on each side produced the two adjacent diverticula. Oka (1892) records that in the Botryllus colonies found along Japanese coasts it is not unusual to find zooids producing as many as six asexual buds. If an even number of buds is produced, there will be the same number of buds on each side of the body; if an odd number is formed, there is always one more on the right than on the left. According to Berrill (1941c) double buds are more likely to develop in early generations of zooids before sexual reproduction begins as well as in later generations when large zooids capable of reproducing sexually are present, rather than in intermediate stages.

Thus it is obvious that there are bilateral bud primordia in both the oozooids and blastozooids, but that there is an asymmetry of budding capacity with the right side usually the more productive. Most of the above information concerning the budding pattern is based on isolated observations or on a study of a very small number of colonies. It seems worthwhile to follow the budding patterns of the colonies studied in this paper in an attempt to determine whether any generalities concerning the budding pattern can be formulated.

When all the colonies are examined on the sixth day of development 6 possess only one 2Z bud (2ZR), 21 possess two 2Z buds (2ZR and 2ZL), and 12 possess three 2Z buds, two on the right side (2ZR and 2ZR′) and one on the left (2ZL). When the second generation zooids are examined on the tenth day to determine the number of 3Z buds per zooid, it is noted that in some colonies each zooid produces only one 3Z bud; in others some zooids produce one 3Z bud and others two such buds; in other colonies all zooids produce two buds each; in still others some zooids produce two 3Z buds and others three 3Z buds. In order to decide whether the number of 3Z buds per zooid in any given colony bears any relationship whatsoever to the number of 2Z buds per zooid earlier produced by the same colony, the data may be arranged advantageously in a checkerboard table (Table II). From this table it is evident that three of the six colonies (50 per cent) which produced only one 2Z bud per zooid also produce only one 3Z bud per zooid; moreover, an additional one of these six colonies (17 per cent) produces one 3Z bud per zooid in at least some of the zooids. Ten of the 21 colonies (48

TABLE II

Comparison of the number of buds per zooid in different generations of the same colonies

		No. of 3Z buds per zooid on tenth day				No. of 4Z buds per zooid on seventeenth day					Av. no. of 6Z zooids per colony on twenty-ninth day
		1 bud	1 bud or 2 buds	2 buds	2 buds or 3 buds	1 bud	1 bud or 2 buds	2 buds	2 buds or 3 buds	3 buds	
No. of 2Z buds per zooid on sixth day	1 bud	3 (50%)	1 (17%)	2 (33%)		1 (17%)	2 (33%)	3 (50%)			6.17
	2 buds	5 (24%)	3 (14%)	10 (48%)	3 (14%)	2 (9.5%)	10 (48%)	8 (38%)		1 (5%)	12.23
	3 buds		7 (58%)	2 (17%)	3 (25%)	3 (25%)	7 (58%)	1 (8%)	1 (8%)		20.90

per cent) which produced two 2Z buds per zooid also produce two 3Z buds per zooid; moreover, six additional colonies (28 per cent) produce two 3Z buds per zooid in at least some of their zooids. Thus, in these two groups of colonies there appears to be at least some slight tendency for colonies to produce the same number of second and third generation buds per zooid. However, those 12 colonies which originally produced three 2Z buds per zooid appear incapable of maintaining this rapid rate of budding, and tend to produce only two 3Z buds per zooid instead (in 17 per cent of these colonies there are two 3Z buds on each zooid, but in 58 per cent there are two 3Z buds on only some zooids and one 3Z bud on others, and in 25 per cent there are two 3Z buds on some zooids and three 3Z buds on others). Pizon (1899a) has noted a similar tendency towards reduction in the number of buds per zooid following a generation in which three buds per zooid was the rule, and he suggests that this reduction serves to compensate for the excessive budding of the earlier generation. In his case the reduction was to even less than two buds per zooid.

Similarly, in order to see whether the number of 4Z buds per zooid bears any relationship whatsoever to the number of 2Z buds per zooid produced by the same colonies these data may be added to the checkerboard table. In some colonies on the seventeenth day there is only a single 4Z bud per zooid; in others some zooids produce one bud, others two; in some colonies each zooid produces two 4Z buds; in others some of the zooids produce two 4Z buds, others three 4Z buds; in one colony each zooid produces three 4Z buds. Of the six colonies originally producing one 2Z bud per zooid only one colony (17 per cent) produces a single 4Z bud per zooid. Fifty per cent produce two buds per zooid, and an additional two colonies (33 per cent) produce two buds per zooid in at least some of their zooids. Thus, by the fourth generation produced by asexual budding these colonies appear to be approaching the ability to produce two buds per zooid. However, if each 4Z zooid then produced two 5Z buds, and each of these in turn produced two 6Z buds, these six colonies should average eight zooids in the sixth generation. Instead they actually average only 6.17 zooids, and therefore slightly less than two buds per zooid must be the rule in giving rise to fifth and sixth generation zooids.

Of the 21 colonies originally producing two 2Z buds per zooid eight (38 per cent) produce two 4Z buds per zooid, but ten (48 per cent) produce two 4Z buds on only some of their zooids. Thus the ability to produce two buds on each zooid is no longer maintained rigidly in these colonies. If each zooid did continue to produce two buds up to the 6Z generation, we would expect to find thirty-two 6Z zooids. Instead we find on the average only 12.23 6Z zooids, indicating a budding rate of about 1.5 buds per zooid beyond the formation of 3Z buds. Those twelve colonies originally capable of producing three 2Z buds per zooid have almost lost this ability by the fourth generation of blastozooids. Only one colony (8 per cent) produces three 4Z buds in some of its zooids; seven colonies (58 per cent) produce two 4Z buds in some of their zooids and in three colonies (25 per cent) only one 4Z bud per zooid is produced. Thus, in these colonies, less than two buds per zooid now seems to be the rule in producing fourth generation blastozooids. If two buds per zooid continued to form to give rise to 4Z, 5Z and 6Z generations, we would expect to find forty-eight 6Z zooids per colony; instead we find only 20.9 6Z zooids per colony on the average, or roughly a budding rate of 1.5 buds per zooid after the 3Z zooids are established.

Grave (1933) has emphasized that the growth rate of different Botryllus colonies varies greatly even in their normal environment, but he made no attempt to analyze these differences in terms of the number of buds produced per generation of zooids. He has observed one colony which developed from a single larva on June 20 to 3000 individuals on July 20; he states that the number of individuals in this colony doubled every two or three days. In this colony the number of buds per zooid must have been at least two to account for such rapid growth; however, he states explicitly that he selected as examples only the most proliferative colonies; therefore in most colonies the budding rate must be less than two buds per zooids.

Therefore, although colonies differ in the number of 2Z buds produced per zooid, this difference appears to be only temporary and is not maintained in later generations, at least not under laboratory conditions. Instead, in later generations all colonies tend to produce on the average 1.5 buds per zooid. Nevertheless, the initial difference in the number of 2Z buds per zooid is sufficiently great that even though the budding rate then tends to become equalized in all colonies, the number of zooids present in the 6Z generation will be small in colonies which originally produced only one 2Z bud per zooid (6.17), will be about twice as great in colonies originally producing two 2Z buds per zooid (12.23), and will be about three times as large in colonies originally producing three 2Z buds per zooid (20.9). Consequently the number of 2Z buds per zooid is an important factor in accounting for differences in the intersiphonal patterns in later generations even when colonies in the same stage of development are compared. The factors which account for the differences in vigor, which in turn allow one, two, or three 2Z buds to form on each zooid, are not known. If they are due to genetical differences the action of heredity in controlling the number of buds per zooid appears to be limited to controlling bud formation only in the establishment of this one generation. These observations seem to be based on a study of more colonies than those examined by Berrill (1935c); his observation that the first bud (1Z) gives rise to *a* second bud (2Z) and that to *a* third bud (3Z) and the third to a bud on each side may hold for a particular colony, but it is certainly not the characteristic budding pattern of the majority of colonies.

Due to premature atrophy of developing buds

In addition to differences in the actual number of buds the possibility that some of these buds may degenerate prematurely before reaching the adult stage must also be considered as a limiting factor in controlling the number of zooids in a colony and therefore the complexity of the intersiphonal patterns of the colony. Pizon (1899a) has observed that frequently a considerable number of buds are arrested in their development and begin to regress. Some disappear very early, at the moment when they are still small simple vesicles which are beginning to produce herniations on the flanks of their parents. Others regress much later when they have already attained one-fifth to one-fourth adult size. Regression even occurs prematurely in some large zooids which have the internal organs almost completely formed.

Pizon (1893, 1899a and 1900) regards the failure of the bud primordia to produce buds as well as the premature atrophy of such buds once they have started to develop as due primarily to nutritive deficiencies. Abortions occur when buds are too closely pressed against the older zooids or against the substrate of the colony, thereby constricting the blood channels and preventing the entrance of the blood stream from parent into bud. Moreover he notes that such atrophy is more frequent in colonies in early stages of formation and suggests that this may be due to the fact that at first all of the energies of the newly forming colony are directed toward elaboration of the test with little left over to enable buds to develop profusely. He particularly emphasizes that isolated zooids or very small systems are more likely to atrophy than large systems, and he suggests that it is necessary that a zooid participate in the organization of a system if it is to survive. Oka (1892) also suggests that crowding or lack of it probably determines whether or not a bud will develop to maturity, but he emphasizes that the left bud primordium is more sensitive to adverse conditions than the right, and is less likely to survive. However, there are other examples of premature degeneration where no apparent causes are to be discerned. During the first seven generations of blastogenesis studied in this present paper there have been few examples of premature atrophy of zooids once they have started to develop. Where such atrophy occurred there was no indication of crowding. Moreover, as noted earlier (p. 83), some zooids degenerate prematurely even after they have become adults and even though they are associated with other zooids within a fairly large system, so participation in system formation is not in itself a guarantee that premature degeneration will not occur.

Due to persistence of parent zooids after their buds attain the adult state

Another factor which can affect the number of zooids present in a colony at a given stage of development is whether the parent zooids always degenerate as soon as their buds become capable of functioning, or whether they sometimes persist to function simultaneously with their buds. The results recorded in this paper are in complete agreement with those of Pizon (1893) who states that when one generation attains the adult state, the preceding generation degenerates, and that two generations of zooids never arrive at the adult state simultaneously. However, several authors (Delle Valle, 1882; Bancroft, 1903; and Berrill, 1935b) have reported that two generations of zooids can function side by side. If this actually does happen, the number of zooids and accordingly the complexity of the intersiphonal patterns would be correspondingly increased.

Due to fusion of adjacent colonies

Yet another factor which could influence the number of zooids in a colony and accordingly the intersiphonal patterns is the possibility that adjacent oozooids or older colonies may fuse indistinguishably. No such fusion was witnessed in the present work because every effort was made to prevent it by destroying adjacent colonies whenever there was a possibility of their fusing. Herdman (1925) suggests that large colonies need not necessarily arise from a single oozooid since several larvae may attach close together and form colonies which fuse so indistinguishably that even within one system some zooids may be derivatives of one oozooid, whereas the others come from another oozooid. Pizon (1900) has recorded an unquestionable case of such fusion. Bancroft (1903) has carried out an extensive study of the process of fusion and he has demonstrated that although unrelated colonies generally do not fuse, fusion can occur between some of the larvae derived from the same mother colony providing they become attached close together. Thus, any colony whose developmental history is unknown may conceivably be the derivative of more than one oozooid.

The Formation and Geometrical Form of Systems and of Intersiphonal Patterns

As indicated in the introduction each system consists of from 2 to 23 blastozooids, with an average of 8.3 blastozooids per system in 200 systems examined in colonies collected at Woods Hole. Obviously the intersiphonal pattern will vary according to the number of zooids involved in its formation. There is no opportunity for a system to form until the second generation zooids are well developed, since only a single zooid is present in the oozooid and first generation blastozooid stages. However, if there are two or more second generation or later generation zooids their atrial extremities approach one another and fusion of the separate atrial openings occurs so that the colony then possesses a central common cloaca about which the zooids are radially arranged with their separate oral siphons at the periphery. The originally separate intersiphonal bands then radiate from the common cloaca and the net result is the first establishment of an intersiphonal pattern. Sometimes, especially in older colonies, the zooids have to move through considerable distance to participate in the establishment of a system. Pizon (1899a) has observed zooids in less than one day make half to almost a complete turn in order to bring their atrial extremities into contact with those of other zooids. Sometimes the formation of a common cloaca occurs in stepwise fashion, i.e., two or more zooids may form one common cloaca, and several may form another, but later these two cloacas fuse to become a single opening. Bancroft (1903) states that there is evidently some attractive force, the nature of which is entirely unknown, which causes zooids to join into a system.

There appears to be a maximum number of zooids which can constitute a single system. According to Pizon (1899b) and Herdman (1925) when blastozooids become too crowded against each other and no space remains for additional zooids around a single cloaca one or more blastozooids become crowded outwards away from the cloaca and come to lie at the periphery, each such isolated zooid constituting a starting point for the formation of an additional system. Oka (1892) also emphasizes that when additional systems are first established they contain only a small number of individuals, usually three or four and many times only two.

Several features of system formation should be analyzed because of the changes in the intersiphonal patterns which are involved. Actually the existence of an intersiphonal band on each blastozooid greatly simplifies the task of observing the relationship of zooids to one another during the formation of systems. a. Is the form of a system (and accordingly of the intersiphonal pattern) identical whenever the same number of zooids forms one system? Is it identical even when different generations of zooids are compared? What factors affect the form of the intersiphonal pattern? b. What is the relationship between the number of zooids in a colony and the number of systems formed? Is there any inherent tendency for a colony to have a certain number of zooids per system, or is the number of zooids comprising a system purely a matter of chance? c. When an additional system is established, does it actually consist at first of a very small number of zooids?

When only two zooids constitute a system they typically occupy less than 90° of the space available around the cloaca and the form of the intersiphonal pattern is invariably the same, viz., the form of a letter V in which the angle between the intersiphonal bands is less than 90°. This is true whether the zooids involved are second, third or fourth generation zooids. Beyond the fourth generation there are usually more than two zooids per system, although in some colonies only a single zooid is present. If three zooids constitute a system the form of the intersiphonal pattern is slightly different depending upon the generation of zooids involved. If the zooids are of the second generation they occupy only 90° of the space available around the cloaca and an angle of approximately 45° is formed between adjacent intersiphonal bands. If the three zooids are third or fourth generation zooids they occupy 180° of the available space around the cloaca and an angle of 90° is formed between intersiphonal bands. If the three zooids involved are fifth or sixth generation zooids they tend to occupy all of the available space around the cloaca and the angle between adjacent intersiphonal bands usually increases to 120°. When systems consist of four zooids they tend to occupy all available space around the cloaca and adjacent intersiphonal bands usually form an angle of 90° with one another, although if third generation zooids are involved, less than 360° of the space is filled and the angle is correspondingly less than 90°. If there are five to ten zooids per system they are arranged radially around the cloaca filling all available space, with equal angles between adjacent intersiphonal bands regardless of the generation under consideration (Pl. I, Figs. 3 and 7). Whenever there are eleven zooids or more in a system the cloaca becomes oval shaped and the system itself is oval in outline instead of circular and the intersiphonal pattern becomes less of a perfect radial pattern (Pl. II, Fig. 9, the system on the right).

Several factors are important in controlling the form of the system. First, whenever possible a zooid tends to be in contact with another zooid on each side and throughout as much of its length as possible. Usually such an arrangement is not possible until more than three zooids are present in a system, because three zooids or less are not usually enough to occupy all of the space around a common cloaca; thus the number of zooids available for system formation is a second factor determining the form of the system. The size of the zooids in the different generations is a third factor involved in the form of a system. Bancroft (1903) and Berrill (1941b and c) have both noted that the size of zooids increases progressively, with each generation. The larger the zooids involved, the fewer needed to occupy all the space available around the cloaca. If the above three factors affecting the form of systems are kept in mind it is possible to predict the form of any system in any colony providing the number of zooids and the generation of zooids is known. These factors exercise full control over the form of the system only when the system is isolated, i.e., only when there is a single system in a colony. When more than one system is present in the same colony the form of the systems is always modified due to crowding of zooids at the contact points between adjacent systems (Pl. I, Figs. 4 and 5).

Certain generalizations can be made concerning the relationship between the number of zooids in a colony and the number of systems formed. The maximum number of zooids which can constitute a single system is most commonly 14 or 15. Occasionally by the time 11 or 12 zooids are present the colony breaks up into two systems. If the colony consists of 16 to 22 zooids two systems are usually formed. If there are 23 to 30 zooids there are usually three systems. When the colony consists of 31 or 32 zooids four systems are usually present. When 52 to 55 zooids are present simultaneously they are most commonly distributed among six systems, and eight systems are usually found when as many as 61 or 62 zooids are present. Whenever the colony consists of more than one system of zooids the actual number of zooids within any one system seems to be determined primarily by chance. On the average there are eight zooids to a system, but there may be from four to fifteen in the colonies whose development has been followed. As far as can be determined there are no inherent differences between colonies in the number of zooids which constitute a system. This means that the number of intersiphonal bands of pigment cells which enter into the formation of any single intersiphonal pattern is purely a matter of chance. It would seem that if each new generation of zooids is larger than the previous one the number of zooids constituting a system should decrease with increased age of the colony. Such a tendency to decrease the number of zooids per system is not evident during the first seven generations of asexual reproduction. However, Bancroft (1903) has noted that in older colonies the number of zooids per system is reduced to three or four on the average.

The observations of Oka (1892), Pizon (1899b) and Herdman (1925) concerning the method of formation of additional systems seem open to question. According to Herdman (pp. 204–205), "When there is no longer room for all the ascidiozooids around the original cloaca, one or more may be pushed out into the surrounding test. Here they continue to form lateral buds and so found new systems." This would suggest that if more zooids are present than the maximum number which can be contained within one system, any extra zooids are crowded out singly from the old system and each of these isolated zooids, by budding, then establishes a new system in the following generation. This implies that in order to form an additional system at least two generations of zooids must be involved: in the first generation one or more zooids become crowded out of the old system; in the second generation the buds developed from the zooids isolated in the previous generation actually establish the new system. It implies, moreover, that at the time an additional system is established the colony always consists of one large system plus the one or more very small newly established systems. Actually any such single zooids if crowded out of a system probably degenerate prematurely (see p. 96). Oka also emphasizes (p. 543) that "Ein neu gebildetes System enthält nur eine geringe Anzahl von Individuen, gewöhnlich deren drei oder vier, manchmal sogar nur zwei. Da aber bei jedem Auftreten einer neuen Generationen die Zahl der Individuen sich verdoppelt, so kommt es im Verlauf einiger Generationen zu einem typischen System von acht oder neun Individuen. . . ." However, elsewhere in his description Oka states (p. 542), "Nach dem Tod der Muttergeneration, welcher bald erfolgt, sieht man die jüngeren in ein System, häufiger aber noch in zwei gleich grosse oder ungleich grosse Systeme sich zusammenlagern." This latter statement of Oka alone accurately describes the formation of additional systems. Even before the death of the parent zooids the buds are roughly grouped according to the systems they will later form. And following the death of the parent zooids the newly formed zooids almost immediately become distributed into as many systems as are destined to form. There is no intermediate step during which all zooids first attempt to join around a single cloaca, followed by isolation of any extra zooids to establish smaller systems. Moreover, an additional system when it is first formed consists of a considerable number of zooids, Oka and Herdman to the contrary. In the ten colonies studied most extensively in this paper an additional system never contained fewer than four zooids when it was first established, and much more commonly it contained seven to nine zooids. Thus, when a colony contains sufficient zooids to form an additional system, the new system does not contain only the excess zooids which can no longer be accommodated in the old system, but rather the old system is replaced by two systems, each with approximately equal numbers of zooids.

Intersiphonal Bands as Taxonomic Characters

Any attempt to use slight differences in the appearance of intersiphonal bands as a basis for classifying Botryllus into species should be regarded with caution. Unless the taxonomist is fully aware of the progressive transformations which the intersiphonal bands undergo and of the time relationship between these transformations and the stages of asexual reproduction errors could be introduced by comparing the appearance of intersiphonal bands in colonies in entirely different stages of development. Moreover, even when colonies of the same stage of development are compared there will be differences in the number of intersiphonal bands in an intersiphonal pattern, as well as differences in the number of intersiphonal patterns in the colony, but such differences are due seemingly to factors which are not of taxonomic importance. And even the absence of intersiphonal bands in some colonies may indicate nothing more than a single gene difference between these and colonies which exhibit intersiphonal bands.

SUMMARY

1. The most striking feature of many Botryllus colonies is the localization of special light-reflecting pigment cells between the oral and atrial siphons of each zooid. Collectively these pigment cells constitute an intersiphonal band.

2. Individual zooids of a Botryllus colony are distributed radially around a common cloaca as a system of zooids. Consequently all the intersiphonal bands within one system constitute a star-shaped pattern called an intersiphonal pattern.

3. Any Botryllus colony is characterized by a specific intersiphonal pattern for only a very brief period of its existence. Any given intersiphonal pattern is then totally destroyed within a few hours and is replaced by an entirely different pattern or group of patterns. The formation and subsequent destruction of intersiphonal bands is described in detail for the oozooid and first seven generations of blastozooids in living colonies established from isolated larvae.

4. The progressive remodeling of the intersiphonal patterns is intimately associated with the changes involved in asexual reproduction. An interval of four to five days occurs between the maximum development of one intersiphonal pattern and the maximum development of the next. During this brief period a typical series of changes occurs in the appearance of a colony. a. The zooids become highly sensitive and contractile and the intersiphonal pattern becomes very irregular. b. Zooids then undergo maximum and permanent contraction, the intersiphonal bands disintegrate and the pigment cells are released into the circulatory system and are carried passively by the blood stream. c. Simultaneously the new generation of zooids is growing and the new intersiphonal bands form in them. At first these are solid bands, but they tend to split lengthwise until they become double intersiphonal bands, especially in older generations of zooids. d. Reflecting pigment cells then disappear from the circulatory system.

5. There is no evidence that the pigment cells released by the break-down of the intersiphonal bands of one generation of zooids can be utilized in the formation of new bands in the next generation of zooids.

6. If the oozooid develops a *strong* intersiphonal band of pigment cells (i.e., a broad, almost solid band of reflecting cells between the two siphons) only *strong* intersiphonal bands appear on all later generations of zooids; if it develops a *weak* intersiphonal band (i.e., only a few reflecting cells near the dorsal midline with no obvious arrangement into a band) only *weak* bands appear on all later generations of zooids. This difference between *weak* and *strong* expression is constant and is probably genetically determined.

7. Intersiphonal patterns vary in different colonies even though they are in the same stage of development. These variations are discussed in terms of a. differences in the number of buds per zooid in different colonies, b. the premature atrophy of developing buds in some colonies, c. the possible functional persistence of parent zooids after their buds become functional, d. the fusion of adjacent colonies.

8. The form of an intersiphonal pattern is determined by several factors: a. Whenever possible zooids tend to come into contact with another zooid on each

side and throughout as much of their length as possible; b. the number of zooids available for system formation is a determining factor; c. the size of the individual zooids likewise affects the form of the pattern. If these three factors are kept in mind it is possible to predict the form of any intersiphonal pattern providing the number of zooids and the generation of zooids is known. When more than one system is present in the same colony the form of the intersiphonal pattern is always modified due to crowding of zooids at the contact points between adjacent systems.

9. When a colony contains sufficient zooids to form more than one system the new system does not contain only the few excess zooids which can no longer be accommodated by the old, but rather the old system is replaced by two systems, each with approximately equal numbers of zooids. As far as can be determined there are no inherent differences between colonies in the number of zooids which constitute a system.

10. The appearance of the intersiphonal bands or patterns should never be used as a characteristic for classifying Botryllus into species unless one is fully aware of the range of variation that occurs even in the same colony and of the factors responsible for such variations.

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[1] For explanation of symbols see Supplement, vol. 84, no. 1, February 1943.

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THE SIZE OF THE MESH OPENINGS IN MUCOUS FEEDING NETS OF MARINE ANIMALS

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Introduction

In a former article (MacGinitie, 1937) the statement was made that "the slime net (Urechis) will entrap the smallest particles which are visible with the aid of an oil immersion lens." Experiments have just been completed which have determined much more accurately the size of the openings through which sea water is passed to strain from it the food ingested by animals using such nets. The results of these experiments have more than justified the statement quoted.

The Feeding Nets

The mucous net of the echiuroid worm *Urechis caupo* (Fisher and MacGinitie, 1928; MacGinitie, 1935) is admirably suited to tests of the kind herein reported. In the mud flats of the west coast Urechis builds a U-shaped tunnel with two entrances eighteen to thirty-six inches apart, depending on the size and age of the animal, and with the lowest part of the tunnel from ten to eighteen inches beneath the surface. The openings of the burrows are somewhat constricted, and the animals, which live singly in a burrow, keep a current of water flowing through the burrow along the body by means of a more or less rhythmic peristalsis.

The slime tube for collecting food is secreted by a ring of mucous glands situated about one-sixth of the length of the body posterior to the proboscis. When the worm secretes a mucous tube, this ring of glands is pressed tightly against the periphery of the burrow to cause the mucus at the beginning of secretion to adhere to the sides of the burrow. As the animal backs up in its burrow more mucus is spun until the tube becomes four to six inches long. Then the spinning ceases, but the tube is allowed to remain attached to the secreting ring. This leaves the animal with its anterior end within a sack of mucus which is firmly attached to the "neck" as though by a drawstring; and the distal end of the mucous sack is firmly attached to the sides of the burrow. Thus all water flowing through the burrow must pass in at the open distal end and pass through the walls of the mucous sack before it can flow along the outside of the animal on its way through the burrow. As the water passes through this mucous net all microscopic and, as will be seen, some ultramicroscopic, material is held by the net. After pumping water through the net for varying lengths of time, the end at the "neck" is slipped over the head and the entire tube with its load of food is swallowed.

The mucous food straining bag of the annelid *Chaetopterus variopedatus* (MacGinitie, 1939a) was also used for the purpose of testing size openings, though Chaetopterus is much more sensitive to strange substances than is Urechis. By using a liberal amount of time and patience, either animal becomes accustomed to the presence of proteins as they are introduced into the water currents passing through the burrows.

MATERIALS AND METHOD

Three substances of known molecular weight, and stained with T1824 Evan's Blue, were introduced into the mucous nets of both *Chaetopterus variopedatus* and *Urechis caupo* while the animals were feeding. The three substances and their molecular weights were: ovalbumin, 44,000; human serum globulin, 176,000; and hemocyanin (Palinurus), 450,000. The molecular weights given were taken from Cohn and Edsall (1943).

In the cases where excess dye was used for staining, the excess was taken up by dialyzing bags (Rawson, 1942–43), the proteins having been dissolved in sea water, 34 parts per thousand of salts. Rawson found that up to eight molecules of dye were bound by each molecule of globulin and that a 1 : 5 solution remained stable for one month. These figures were used also for ovalbumin and hemocyanin, though no experimental evidence was found for the use of eight molecules as a saturation limit, but absorption by cellophane dialzying bags showed these limits to be fairly accurate.

RESULTS

The ovalbumin passed through the mucous net entirely. Enough serum globulin was held by the net to color it a decided blue, while no hemocyanin whatever passed through the mucus.

Molecular weights and shapes of proteins are subject to considerable uncertainty. Recently Cohn (1944) gives a molecular weight, length and equatorial diameter for human serum globulin of 160,000,320 Å, and 36 Å, respectively. The a/b ratio is thus about 1/9. Human serum globulin has a frictional flow coefficient of 1.49 and hemocyanin of 1.23. Comparison of the two molecules on this basis and disregarding hydration, gives hemocyanin an equatorial diameter of approximately 90Å, an a/b ratio of 1/5.

Ninety Ångströms seems to be a rather high figure, judging by what one can find in the literature relative to the diameters of protein molecules, but it is safe to say that the meshwork opening in a mucous feeding net is of a magnitude between 36 Å and 90Å. However, since some human serum globulin was caught by the net while no ovalbumin was, and because all hemocyanin was entrapped, a logical conclusion is that the meshwork openings are quite close to 40 Å.

Solutions varying from two molecules of dye to one of protein to those with an excess of dye were used with hemocyanin. Since the dye T1824 has a molecular weight of 960 (Rawson, 1942–43), the molecular weight of any of the substances used was not increased by more than 7,680. Human serum globulin was used in the proportion of five molecules of dye to one of the serum; thus the molecular weight was increased by 4,800. Allowing for this increase, the figure 40 Å was used instead of 36 Å, though the latter may be the more accurate figure.

The results described in this paper were the same for the mucous nets of both Chaetopterus and Urechis, and it is my opinion that all feeding nets where used by tunicates (MacGinitie, 1939b), pelecypods (MacGinitie, 1941), gastropods (in mss.), etc., are of like physical and chemical structure, though only actual tests with each animal will prove this to be true.

Discussion

There are several factors which preclude exact measurements, for example, uncertainty of molecular size, the shape of molecules, hydration, the shape of openings in the mucous network, surface conditions of both protein and mucoid molecules, rigidity of the mucus surrounding the openings, force or head of water flow, possible variation in the elasticity of the mucus, etc.

Molecular size should not show an error of more than plus or minus ten per cent or less, and the shape of molecules about the same or possibly more. It is difficult to conceive of straight openings from side to side of a mucous net .5 to 1 mm. in thickness. Where the mucous net remains attached to the body of Urechis it is about 3 mm. in thickness, and it was noted that the hemocyanin did not penetrate greatly beyond the inner surface. It did penetrate sufficiently, however, to become incorporated in the mucous network. It cannot be washed out, and later it is shown that no reaction takes place between the mucous tube and the hemocyanin. This indicates a sponge-like structure, and not openings running directly from side to side. Mehl (verbal communication) is of the opinion that the mucous molecules are long and slender. The last three factors, rigidity, force of flow and variation in elasticity, are more interdependent. The greater the rigidity, the more uniformly constant the openings, and the less affected by force of current.

Mucous nets are held under a certain amount of tension while the animal is feeding. When the net of Urechis is freed from the animal it at once collapses to one-half its original size and gradually draws up to much less than this if left free and immersed in sea water.

When Urechis is pumping water through a burrow of glass tubing for respiratory purposes (not feeding), and one end of the tube is held out of water the peristaltic movements can create a difference in head of four or more inches, which is equal to .144 lb. per square inch. Such pressures are never exerted upon a feeding tube. Under natural conditions perhaps but one-tenth of this amount of pressure is exerted upon the feeding tube.

A check was made to determine if on standing there was any reaction between the protein of the mucous tube and that of the substances used. Clean mucous nets were put in stained solutions of hemocyanin and human serum globulin and left for one-half hour periods, then centrifuged; no hemocyanin or serum globulin united with the tubes.

No dye has yet been found that will stain the pure mucus of a feeding net.

In one rechecking of these experiments, some hemocyanin (Palinurus) was used which had been precipitated by low pH and stored in a frozen state for about six months. This hemocyanin had denatured to the extent that it all passed through the mucous net. It is known that hemocyanin denatures to a greater extent than most proteins, though all hemocyanins are not alike in their amount of dissociation. Neurath, Greenstein, Putnam and Erickson (1944), quoting Burk (1940), give a dissociation for Limulus hemocyanin of from 2,040,000 to 142,000 for 6.66 M urea treatment and to 69,000 for HCl followed by 6.66 M urea. Measurements were made on the basis of osmotic pressures. This is a dissociation to about 1/14 for the urea treatment and 1/30 for the combined HCl-urea treatment. The difference between 142,000 and 69,000 is due to the removal of copper by the acid.

The fact that all of the hemocyanin used in this rechecking experiment passed through is proof that its molecular size had been reduced, at least in one dimension, by a low pH or by freezing, to a size lower than that of the human serum globulin.

Another factor, of which there are perhaps many more than were listed above, that could influence the passage of long molecules through a network such as that just described is the possibility that by physical means the molecules may be oriented in such a way that they may enter the network openings of the mucus small end first. This could be due to Brownian movement or electrical charge or flow influence. Perhaps these feeding nets may be of value in helping us to solve such problems.

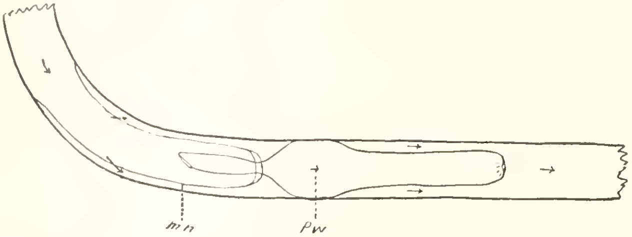

Diagram of *Urechis caupo* in a burrow, feeding. *m.n.*, mucous net; *p.w.*, peristaltic wave which drives water ahead of it. Other arrows indicate flow of water through the burrow.

The mucous net has been used to estimate the extent of polymerization in certain proteins. It already has been shown that it is a test for denaturation of hemocyanin (Palinurus). It will also be useful in checking partial dissociation or denaturation or for possible adulteration by smaller protein molecules.

There still remains the possibility that other animals may secrete mucous nets with meshwork different in porosity from that of Urechis and Chaetopterus, and further experimentation may show that the mucous net may be stretched or allowed to contract to further increase its range of usefulness.

Considering the above findings, one is impressed with the efficiency of mucus as a net for straining food from water. The molecular size of decomposition products would preclude their use as food material, but all other organic materials, including protein molecules of a size greater than 40 Å in cross section could be used.

It is interesting to think about a substance, which when it comes in contact with water, takes up 200 or more parts by weight of the latter, yet remains a meshwork which allows water to pass freely through it. It is also interesting that in its wide use among the marine invertebrates as a food gathering mechanism it is seldom if ever wasted, since it is necessarily swallowed with the food that it has entrapped.

I wish to express my appreciation to Dr. Linus Pauling, Dr. Dan H. Campbell and Dr. Albert Tyler for advice and materials, and to Dr. T. H. Morgan for reading the manuscript.

SUMMARY

1. A brief description is given of the food collecting net of the echiuroid worm *Urechis caupo*. Water flows freely through this mucous net.

2. By introducing into this mucous tube proteins of known molecular size, namely, ovalbumin, 44,000; human serum globulin, 176,000; and hemocyanin (Palinurus), 450,000, the size of the openings was found to be approximately 40 Ångströms.

3. Experimental uses for the mucous net are given, and other possible uses suggested.

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STUDIES ON THE OXYGEN CONSUMPTION AND WATER METABOLISM OF TURTLE EMBRYOS[1]

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As a result of the early work of Bohr and Hasselbalch and the later experiments of Murray and others there is available a considerable body of information concerning the respiratory exchange of the chick throughout its embryonic life and, although vertebrate groups other than birds have been less extensively investigated, the respiratory characteristics of the embryos of various fishes and amphibians are also fairly well known. Only two investigations of the respiration of reptilian embryos have been reported however, and these deal with but a few stages.

Bohr (1904) carried on some experiments with the eggs of the snake, *Coluber natrix,* and found a decrease in respiration intensity with increasing age. The respiratory quotient as determined by Bohr was about 0.9 at all of the stages studied.

Zarrow and Pomerat (1937) studied four eggs of the Green Snake (*Liopeltis vernalis*) which were in late stages of development and also investigated the respiration of the young after hatching. They report that the absolute gas exchange of the eggs was of the same order of magnitude as that of the young snakes but the respiratory quotient of the hatched animals was significantly greater than that of the embryos.

The present experiments were undertaken with the purpose of providing closely spaced series of determinations of oxygen consumption of the reptilian embryo from the time the egg is laid until after hatching. Some data on changes in weight of the egg during development and on dry substance and fat content at different stages are also presented.

MATERIAL AND METHODS

The eggs used in these experiments were obtained from four species of turtles; the Common Mud Turtle (*Kinosternon subrurum subrurum*), the Snapping Turtle (*Chelydra serpentina serpentina*), the Three-toed Box Turtle (*Terrapene carolina triunguis*), and the Eastern Painted Turtle (*Chrysemys picta picta*). One of these, the Three-toed Box Turtle, is a terrestrial form while the others are primarily aquatic. The Mud Turtles and Painted Turtles were collected from ponds in nearby Maryland, the Snapping Turtles were purchased from a dealer in Wisconsin and the Three-toed Box Turtles were sent to us from Monticello County, Arkansas, through the courtesy of Dr. Delzie Demaree.

Eggs were obtained by killing the adult females and removing the eggs from the oviducts. They were kept on moist cotton in open dishes in an incubator the temperature of which varied between 25.0° and 25.5° C. Most of the eggs maintained under these conditions seem to have developed normally although a number of them died during the course of the long period which elapsed before hatching. Dead eggs had a tendency to become moldy on the surface and were, of course, discarded. Moreover it proved possible after some experience to ascertain, by candling the eggs, whether or not living embryos were present and in all experiments except those with very early stages this test was employed. A further check was provided by opening some of the eggs of the experimental series from time to time or by carrying them through to hatching. It is thus possible to state positively that all of the eggs which provided the data on oxygen consumption were living and apparently developing normally.

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[1] We are indebted to the Permanent Science Fund of the American Academy of Arts and Sciences for a grant for the purchase of the Warburg vessels used in this investigation. The water bath used was made available through a previous grant from the Elizabeth Thompson Science Fund.

To obtain some information concerning changes in weight during development, some eggs of each species were weighed at intervals on an analytical balance. In all cases excess moisture was wiped from the surface before weighing and weights were taken to the nearest milligram.

Determinations of the oxygen consumption of eggs and hatched turtles were carried out with Warburg manometers equipped with flasks having about 120 cc. capacity. The CO_2 was absorbed in the customary manner with KOH, the flasks were not shaken and the eggs were kept dry. In all experiments seven flasks were used simultaneously, one serving as a thermobarometer while the other six contained eggs. In the great majority of the experiments only a single egg was introduced into a flask. With very young stages at the beginning of our study, several eggs were used in each flask. Since, however, not all eggs develop normally it was later necessary to reject some of these experiments because, upon opening the eggs at a later date, one or more of a lot used in a single manometer proved to be infertile or abnormal. Since the rate of oxygen consumption of young stages is low, while that of older ones is high, the individual determinations were carried through periods of various lengths. With very young stages the usual period was 24 hours with two or three readings only, with older stages the period was 5 to 6 hours with hourly readings and with late stages 2 to 3 hours with half-hourly readings. In the case of very late stages of the Snapping Turtle the experimental periods were sometimes limited to 1½ hours because the oxygen consumption was so high as to preclude further readings with the manometers used. At the outset of the experiments it became clear that the temperature equilibration between the surroundings and the egg is very slow. When eggs were introduced directly from the incubator at 25.0–25.5° C. into the manometer flasks in a water bath at 25.4 ± 0.01° C. the equilibration took at least two hours. It was therefore necessary to keep the eggs that were to be used for an experiment in the water bath over night. This was done by placing them on moist cotton in an open jar immersed in the water bath. Under such conditions an equilibration of 45 minutes before the manometers were closed proved to be sufficient.

Since it was necessary, in making the calculations of oxygen consumption, to know the volumes of the eggs used, a method was devised for measuring the volumes to the nearest 0.1 cc. The apparatus employed for this [2] was a wide-mouthed, glass-stoppered bottle with a 10 cc. graduated pipette fused in the stopper and a 20 cc. graduated syringe fused in the side of the bottle near its base. The bottle was filled with water, closed and the water forced into the pipette by pushing the syringe to the zero mark. A reading was then taken on the pipette. The syringe was opened again to lower the water level, the stopper of the bottle was removed and the egg to be measured was inserted. The top was then replaced, the syringe was pushed to the zero mark and a new reading was taken on the pipette. The difference between this reading and the preceding gives the volume of the egg.

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[2] We are indebted to Mr. W. F. Simpson of this department for devising this apparatus.

Determinations of the dry substance and fat content were carried out as follows. Two batches of young Snapper eggs (ten eggs each) and two batches of newly hatched Snappers (ten and seven turtles respectively) were dried to constant weight in an oven at 100–105° C. In the case of the eggs, the egg contents and the shells were dried separately. The fat content was determined according to the method of Kumagava and Suto (1908) with the slight modification used by Reinhard and von Brand (1944).

Large numbers of eggs of the Painted Turtle and Snapping Turtle were available and eggs of these forms were opened every few days to give closely staged series of preserved embryos. This makes it possible to correlate the oxygen consumption with both the age and the stage of development in these species. From the Mud Turtle and the Three-toed Box Turtle relatively few eggs were obtained and our series of normal stages for these animals are therefore incomplete.

RESULTS

Developmental rate and incubation period

In view of the paucity of published information concerning the embryology of the turtles used in the present work it is necessary, before discussing our experimental results, to give some general account of the developmental history of these forms.

The normal egg complement of a single female differs markedly in the four species. For the Mud Turtle we had only two adult females and these yielded four and three eggs respectively. Six adult Three-toed Box Turtles were used; four of these had only two eggs each in the oviducts and the other two had three eggs each. In a group of ten Painted Turtles the number of oviductal eggs ranged from two to eight, but most individuals had either five or six eggs and the average number was 5.4. The Painted Turtles used were all about the same size, having carapace lengths of 13.0 to 15.5 cm. Nine Snapping Turtles with carapaces 27.0 to 33.0 cm. long were opened. The smallest number of eggs found was 29, the greatest 61. The average yield for the nine animals was 44.5. This is of some interest in view of the fact that Pope (1939) records for the Snapper that "A female lays from twenty to about forty eggs at one time. Higher numbers (even up to seventy) are often given, but they probably are either sheer estimates or counts made in compound nests." It is, of course, not certain that all of the eggs found in the oviducts would be laid at one time or in one nest but our records do show that a single female may produce well over 40 eggs during one season.

In all of these species, and probably in turtles generally (Risley 1932, 1944), the eggs pass through the cleavage stages while still within the oviduct and are in early stages of gastrulation when laid. If the eggs are retained in the oviduct for unusually long periods they practically cease development during this time, possibly because of anaerobic conditions, and therefore eggs removed directly from the oviduct are all at the same stage even though the dates of removal may be several weeks apart. Thus in our experiments, from a single lot of Snapping Turtles some females were killed on June 30 while others were retained until July 27, but the eggs of both were at the same stage of development when removed and were carried through to hatching with equal success.

At the temperature used in the present work (25.0–25.5° C.) the average times required for incubation were as follows: Mud Turtle, 76 days; Painted Turtle, 63 days; Snapping Turtle, 72 days. None of the Three-toed Box Turtle eggs were carried through to hatching but eggs opened after 70 days incubation contained well-formed turtles attached to yolk masses about 6.0 mm. in diameter. They would probably have required at least 10 more days before hatching. It should be pointed out that even under uniform environmental conditions there is some variation in the rate of development and the period of incubation. For example, in a single clutch of 8 Painted Turtle eggs the incubation period varied from 58 to 68 days and in a clutch of 61 Snapping Turtle eggs the limits of variation were 69 to 78 days.

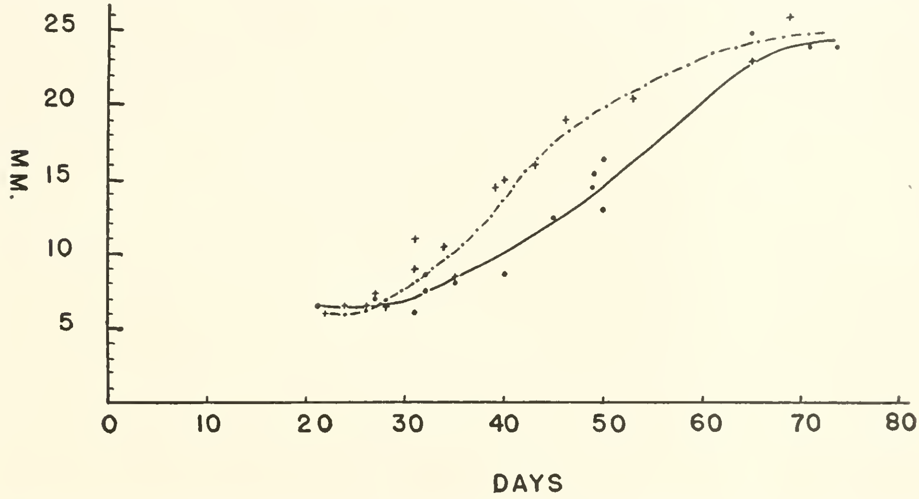

FIGURE 1. The increase in length of the carapace of the turtle embryo during the period of development. ● = Painted Turtle; + = Snapping Turtle.

As an indication of the relative rate of growth of the embryo at successive stages, measurements have been made of the series of preserved embryos of the Painted Turtle and the Snapper. Because of the difficulty of making reliable measurements of total length in these animals it has seemed best, for the present purpose, to use the length of the carapace. This can be measured accurately and does seem to furnish a reliable criterion of the rate of growth of the body as a whole. Such a procedure necessitates neglecting about the first twenty days of development, before the carapace is formed, but as will be seen it is the latter part of development which is of most interest in our results on the oxygen consumption.

Figure 1 gives curves for the increase in length of the carapace in the Painted Turtle and Snapping Turtle. Each point represents a single measurement. In the case of the Painted Turtle all embryos in the series were from eggs kept in the incubator. Because of lack of space, the Snapper eggs used for the preserved series were kept in a laboratory the temperature of which varied but averaged about 25° C. The fact that these eggs hatched at the same time as did those in the experimental series of Snapper eggs which were kept in the incubator seems to justify the conclusion that the growth rate in the two lots was quite similar.

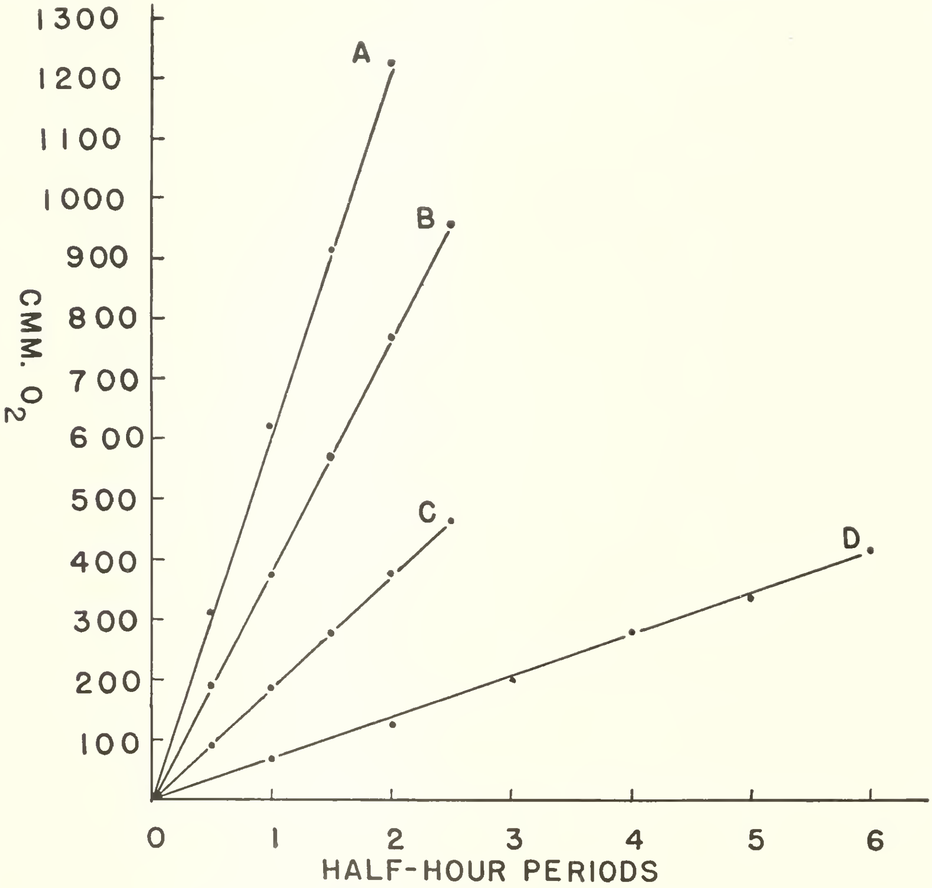

FIGURE 2. Oxygen consumption of four different specimens during consecutive periods of single experiments. A = newly hatched Snapping Turtle; B = Snapping Turtle egg, 42 days incubation; C = Mud Turtle egg, 45 days incubation; D = Painted Turtle egg, 23 days incubation.

It will be noted that the curves for the two species agree closely and indicate that the growth rate is relatively low during the early stages of development but rises rapidly after about the thirtieth day and then tends to remain nearly constant after the sixtieth day. The growth rate of the Snapping Turtle is somewhat higher than that of the Painted Turtle during the period from the thirtieth to fiftieth day however, so that its curve lies at a higher level than does that of the Painted Turtle during this time. The general similarity of these curves to those for oxygen consumption will be noted later.

It should be pointed out that, although the length of the carapace at hatching was found to be the same in the Painted Turtle and Snapping Turtle, the Snapper hatchling is really much the larger animal for it is broader and bulkier than the young Painted Turtle. Thus the average weight of ten new-hatched Painted Turtles was 4.25 grams while the average weight of ten Snapper hatchlings was 7.18 grams. Clearly, therefore, the measurements of carapace length serve only

TABLE I

Oxygen consumption of developing turtle eggs, turtles in the process of hatching (hg) and newly hatched turtles (h). The figures are mean values representing cubic millimeters of oxygen per egg or organism per hour.

Age in days	Three-toed Box Turtle	Mud Turtle	Painted Turtle	Snapping Turtle
1			18	
2	14		32	
9	29	13		
11				35
12		12		
15			39	
16		21		40
19	51			
23		31		55
24	80			
32			95	
34		82		109
35	136			
37			133	
41			185	
42				332
44	180			
45		171		
49			293	
50	287		375	
52				600
54	349	386	526	
56			456	
57			396	
61			463	
62		387	552	798
63	428		632 (hg)	
64			427	
65			476	
69			555	751
70	350	447		1,036 (hg)
72				868 (hg) 641 (h)
73			581	
75		418		617 (h)
76		517 (h)	607 (h)	795 (h)
81			748 (h)	
83		437 (h)		
90		541 (h)		

to give an indication of relative growth rates at different periods and do not tell us the absolute rate of growth. Since, however, our primary interest in relation to the oxygen consumption experiments is in the relative rate of growth, the data derived from the length measurements seem sufficient for our purpose.

Oxygen consumption

When the precautions outlined in the section on material and methods were taken, the successive readings of single manometers proved to be quite regular regardless of the stage of development of the contained egg or young turtle. Examples of the actual oxygen consumption during individual experiments taken at random from our protocols are shown in Figure 2 to illustrate the various lengths of experimental periods, the various times between readings and the obvious regularity of the oxygen consumption.

The mean values of all our experiments on the oxygen consumption of the turtle egg are presented in Table I. The number of individual determinations at each stage varied somewhat but most of the figures in this table are averages of from three to six determinations. These same data are shown graphically in Figures 3 and 4. The figures for the Mud Turtle, Painted Turtle and Three-toed Box Turtle were fairly close, if identical ages are compared, and they fit rather well to one curve (Fig. 3). The egg of the Snapping Turtle, as was to be expected in view of its greater size, proved to have a much higher oxygen consumption than did the eggs of the other three species (Fig. 4). It has been pointed out that the weight of a newly hatched Painted Turtle is only about 60 per cent of that of a Snapper hatchling and it may be assumed that such weight differences exist even at early embryonic stages. Despite the fact that the curve for the Snapper is at a higher level than that for the other three species, it is obvious that the fundamental nature of the curve is the same in all the forms investigated. Its general shape corresponds to that of the respiratory curves described for the hen's egg (Bohr

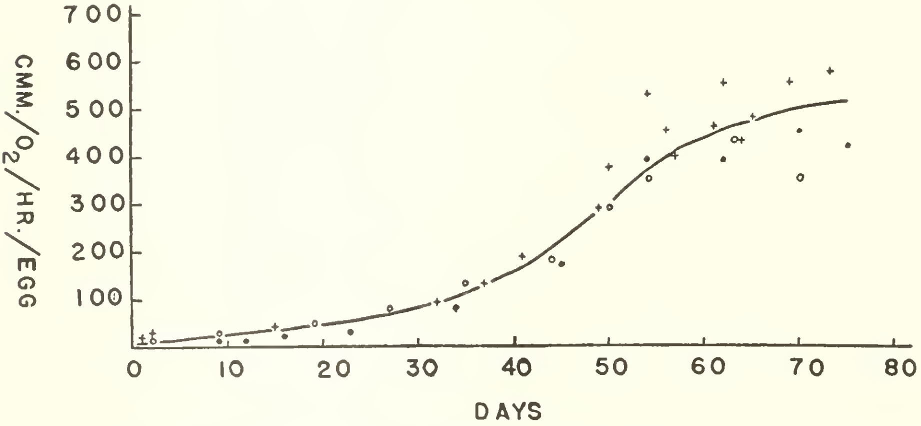

FIGURE 3. Oxygen consumption of the developing eggs of the Mud Turtle, the Painted Turtle and the Three-toed Box Turtle during the period of incubation. ● = Mud Turtle, + = Painted Turtle, ○ = Three-toed Box Turtle.

and Hasselbalch 1900, 1903; Murray 1925). It should be noted that the curve for the oxygen consumption of the Snapper egg shows a somewhat steeper slope during the period between the thirtieth and fiftieth days than does the curve for the other three species. Reference to Figure 1 shows that this must be related to the more rapid increase in size shown by the Snapper embryo during this time. The similarity of the curves for oxygen consumption to those for length increase is quite striking.

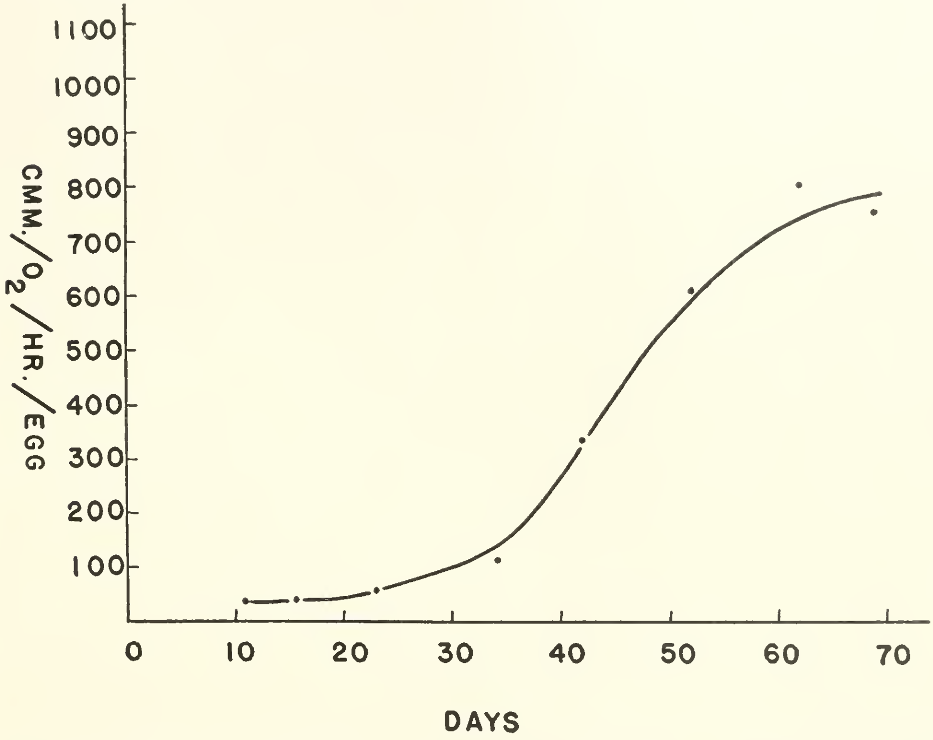

FIGURE 4. Oxygen consumption of the developing eggs of the Snapping Turtle during the period of incubation.

In the case of the turtle one may say that, in general, during the first 50 days of incubation the oxygen consumption is a little less than doubled every 10 days. The single points deviate from the curve but slightly during the first 40 to 50 days but later the deviations become quite pronounced. This period of irregularity corresponds more or less with the period when the curve flattens out. There is, of course, no doubt that the movements of the embryo increase during the latter part of development and it can be assumed that variations in muscular activity are responsible for the variability in the rates of oxygen consumption of older embryos. That this assumption is correct can be inferred from another observation. In Table I are listed some values for turtles that were in the process of hatching and it will be seen that these values are higher than both those for advanced embryos and those for young hatched specimens. The obvious explanation is that the breaking of the egg shell and emergence from the egg require special muscular efforts and so necessitate a higher oxygen consumption during that time.

It should be noted that the values listed in Table I for the oxygen consumption of hatched turtles are about the same as, or only slightly higher than, those for advanced embryos. This is in line with the observation of Zarrow and Pomerat (1937) on embryos and young of the Green Snake. In the case of the chick however the young, during the first ten days, consumes about five times as much oxygen per hour as does the embryo just before hatching (Lussana 1905). This is doubtless to be ascribed to the great amount of muscular activity of the young chick.

The average oxygen consumption per gram per hour for the hatched turtles were as follows: Mud Turtle, 178 cmm.; Painted Turtle, 186 cmm.; Snapping Turtle, 106 cmm. An exact comparison with rates of oxygen consumption of adult turtles is not possible since no data are available from experiments performed near 25° C. Baldwin's (1926) figures for experiments conducted at 20° C. are: Painted Turtle (*Chrysemys picta marginata*) of 1,200 gm., 49.2 cmm./gm./hr.; Snapping Turtle of 1,700 gm., 56.2 cmm./gm./hr. If, as can be supposed, the temperature coefficient of the turtle respiration is in the normal range of about 2, the oxygen consumption per unit weight of young specimens is somewhat higher than that of adults. This would be in agreement with the findings of many investigators for both cold-blooded and warm-blooded animals. In view of the uncertainty of the temperature coefficient it seems premature to try to calculate whether identical values for juvenile and adult specimens would be obtained if the calculations were based on surface area rather than on weight.

TABLE II

Individual variations in the oxygen consumption of eggs of the same age

Species	Age in days	O_2 consumption in cmm./egg/hour					
Snapping Turtle	24	59	67	39	52	59	
Snapping Turtle	52	806	355	624	592	710	516
Snapping Turtle	62	730	914	553	860	884	845
Three-toed Box Turtle	27	82	73	85			
Three-toed Box Turtle	44	206	153				
Painted Turtle	37	140	144	116			
Painted Turtle	54	493	538	494	565	536	
Painted Turtle	76	645	568	636	581		

In connection with the curves shown in Figures 3 and 4 it is necessary to emphasize that the individual points on the curves represent averages of several determinations and that, in some cases, there were considerable differences in the rate of oxygen consumption of different eggs of the same age. The extent of such variations is indicated by some examples presented in Table II. In most cases we are not able to account for these variations but it is clear that not all eggs developed at exactly the same rate in spite of the uniformity of the external conditions and therefore we occasionally found that two eggs of the same lot, opened on the same day contained embryos which differed significantly in size. For example, two Painted Turtle eggs taken from the same female and incubated for 50 days both contained living and seemingly normal embryos but one embryo measured 13.0 mm. while the other measured 16.5 mm. Similar cases could be cited for the Snapper and the Three-toed Box Turtle. At the later stages of development it is probable that individual differences in the amount of movement of the embryos during the period of the experiment would suffice to account for most of the variations in oxygen consumption. It is, in fact, apparent from our protocols that, during the late stages, a single egg used on successive days often showed a regular, but relatively high rate of oxygen consumption on one day and a regular but low rate on another. There would thus seem to be some periods of activity of fairly long duration and other periods when the embryo is quiescent.

Despite the individual variations discussed above it is clear, from examination of Figures 3 and 4, that the number of determinations and the number of eggs used in these experiments must have been large enough to eliminate any great errors due to variations. Otherwise one would hardly expect such smooth curves.

Changes in weight, dry substance and fat content during development

Previous observations on weight changes in the eggs of turtles have indicated that there is a considerable water intake during development. Cunningham and Hurwitz (1936) report a 37 per cent increase in the weight of the egg of the Diamond-back Terrapin and a 50 per cent increase in the egg of the Marine turtle, *Caretta caretta*. Cunningham and Huene (1938) found that the Snapping Turtle egg may show a maximum weight increase of 60 per cent and the Painted Turtle egg a maximum increase of 75 per cent.

In view of these results Needham (1942) lists the chelonian egg as a non-cleidoic egg, one which is dependent upon uptake of water from the environment for successful incubation. Although this subject was not of immediate concern in the present investigation, some of the eggs of each species were weighed from time to time and the results seem worthy of mention as indicating that the eggs of different species are quite differently constituted in respect to their water requirements and that the amount of water intake necessary for successful development need not be so great as the figures of Cunningham and Huene would indicate. In our experiments the eggs of the Snapping Turtle, the Painted Turtle and the Three-toed Box Turtle all showed an increase in weight during development but the increases were not so great as those reported by Cunningham and Huene. This difference is probably due to a difference in conditions. All of the eggs in our experimental series were kept on cotton which was constantly kept moist and the incubator always had some moisture condensed on the walls. Under these conditions a high percentage of the eggs hatched successfully. On the other hand, eggs kept on moist cotton on a laboratory shelf lost water rapidly, as evidenced by collapsing of the shells, and it was found necessary to keep these eggs covered by a layer of wet cotton in order to bring them through to hatching. The eggs used by Cunningham and Huene were buried in moist sand.

A group of eight Painted Turtle eggs which were kept in the incubator were weighed at approximately weekly intervals throughout their development and the young turtles from these eggs were weighed shortly after hatching. The initial weights, taken within 2 hours after removal from the oviducts, ranged from 4.858 gm. to 5.773 gm. and gave an average of 5.275 gm.; at 60 days incubation the weights ranged from 5.821 gm. to 6.349 gm. and averaged 6.069 gm. There was thus a weight increase of 0.794 gm., approximately 15.1 per cent of the initial weight. It is of interest that this increase was made mostly during the first 9 days of development and that after that time the weights of all eggs stayed fairly constant. The average weight of these eggs 24 hours after removal from the oviducts was 5.286 gm. but at the next weighing (9 days) the average weight was 6.020 gm. The young which hatched from these eggs varied in weight from 3.867 gm. to 4.470 gm. and averaged 4.205 gm.

Our data for the Snapping Turtle and the Three-toed Box Turtle, although not so complete as those for the Painted Turtle, indicate a similar water uptake from the environment. The average weight of ten Snapping Turtle eggs at one day of incubation was 9.944 gm.; at 61 days they had increased in weight to give an average of 10.979 gm., an increase of 10.4 per cent. The average weight of five Three-toed Box Turtle eggs was 8.153 gm. at one day and 9.66 gm. at 70 days, an increase of 18.5 per cent.

Only four eggs of the Mud Turtle were available but these, although they were kept in the same dishes as the Painted Turtle eggs and were weighed at the same times, gave strikingly different results. The four eggs had initial weights of 4.010 to 4.370 gm. and averaged 4.211 grams. The averages for the later weighings were as follows: 9 days, 4.120 gm.; 19 days, 4.144 gm.; 28 days, 4.106 gm.; 38 days, 4.147 gm.; 54 days, 4.120 gm.; 60 days, 4.112 gm. Thus these eggs under the same conditions as those of the Painted Turtle showed no significant change in weight during the whole developmental period. The difference between these two is probably to be attributed to the difference in the nature of the egg shell for the shell of the Mud Turtle egg is hard, thick and brittle while the Painted Turtle egg has a shell which though tough, is more or less parchment-like and can be dented with the finger without breaking. The eggs of the Snapper and Three-toed Turtle have thicker and more rigid shells than do those of the Painted Turtle but do not have the brittle, china-like appearance of Mud Turtle eggs.

In view of the fact that a high percentage of the eggs in our experimental series hatched and gave normal young turtles many of which are still alive we may conclude that the egg of the Mud Turtle is a cleidoic egg in Needham's sense, requiring no uptake of water for its successful development and that the eggs of the other three species used are able to carry through their development with much less water uptake than was previously believed. Just what the minimum requirements may be remains to be investigated.

The initial differences in weight of the eggs of the different species used are, of course, chiefly due to characteristic differences in size of the eggs. In the extensive literature dealing with the natural history of turtles it is customary to indicate these size differences by giving measurements of the length and breadth of the egg but since turtle eggs are often rather irregular in shape we feel that the volume of the egg furnishes a more reliable index of its relative size. The average volumes of the eggs used in the present work were as follows: Mud Turtle, 3.7 cc.; Painted Turtle, 5.3 cc.; Three-toed Box Turtle, 8.1 cc.; Snapping Turtle, 8.7 cc. The limits of individual variation were: Mud Turtle (4 eggs), 3.6–4.0 cc.; Painted Turtle (26 eggs), 4.7–6.1 cc.; Three-toed Box Turtle (12 eggs), 7.1–9.0 cc.; Snapping Turtle (17 eggs), 7.5–9.9 cc. It may be of interest to note that in our largest clutch of Snapping Turtle eggs (61 eggs) there were two which were unusually large, having volumes of 15.3 and 13.5 cc. respectively. These when opened proved to be double-yolked eggs both of which were, unfortunately, infertile. We have seen no other double-yolked turtle eggs nor have we encountered any reference to them in the literature.

Our data on the changes in dry substance and in fat content apply to the Snapping Turtle only and were derived from determinations on 20 eggs (at 15 days incubation) and on 17 newly hatched turtles. The eggs were analyzed in two batches of ten eggs each and the turtles in two batches of ten and seven animals respectively. The results of these determinations are summarized in Table III.

TABLE III

Changes in dry substance, water content and ether extract during the development of the Snapping Turtle egg. The values are expressed in grams per egg (or, in the case of hatched turtles, in grams per individual) and are mean values of two determinations. The single values are given in parentheses.

	Egg contents	Egg shell	Hatched turtles	Decrease during development
Fresh weight	8.80 (8.54, 9.05)	1.56 (1.56, 1.57)	7.36 (7.54, 7.18)	1.44
Dry weight	1.91 (1.89, 1.92)	1.14 (1.15, 1.13)	1.45 (1.52, 1.38)	0.46
Water	6.89 (6.65, 7.13)	0.42 (0.41, 0.44)	5.91 (6.02, 5.80)	0.98
Ether extract	0.273 (0.258, 0.288)		0.212 (0.220, 0.203)	0.061

These data show that the hatched animal has considerably less water than the original egg and about 0.5 gram less dry substance. Much of the water loss is to be accounted for by the fluids of the amniotic and allantoic cavities which are lost at hatching. We have no precise measurements on this for the Snapping Turtle but one of the Mud Turtle eggs was opened on the 77th day and the following weights were obtained: whole egg, 4.341 grams; embryo, 3.207 grams; shell, 0.674 gram; fluid, 0.409 gram; unaccounted for, 0.054 gram. The fluid lost at hatching thus amounted to at least 9.4 per cent of the weight of the whole egg just before hatching. A comparable percentage is undoubtedly lost in the Snapping Turtle. Although no determinations of the inorganic substances were made, it can be assumed that the loss of dry substance was primarily due to the oxidation of organic material.

Fat was consumed to the extent of 61 mg. per egg. Since one gm. of fat of average composition requires 2,020 cc. of oxygen for total oxidation the fat disappearing from the Snapper egg would require 123 cc. of oxygen for its combustion. The total oxygen consumption during development up to hatching, as computed from Figure 4, is about 500 cc. This leaves about 380 cc. of oxygen for oxidation of the 400 mgm. of dry substance additionally consumed (total dry substance used, minus fat consumed). Four hundred mgm. of carbohydrate are totally oxidized by 300 cc. of oxygen and the same amount of protein by 400 cc. of oxygen. It

THE DEVELOPMENTAL HISTORY OF AMAROECIUM CONSTELLATUM. I. EARLY EMBRYONIC DEVELOPMENT

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Introduction

The Tunicata have held the interest of biologists since Kowalevsky established their taxonomic position by identifying the larvae with Chordates. In their embryology apparent mosaic development has presented a rich field of investigation for both descriptive and experimental embryology. Conklin's exhaustive study of Styela (1905) established the pattern of development which has been verified for Ascidiella, Ciona, Molgula, Botryllus, Phallusia, and also for Amphioxus. These forms agree in having a moderate supply of yolk that neither obscures nor interferes with the pattern of development. Studies on the early embryology of heavily yolked forms have been restricted to papers on Distaplia (Davidoff, 1899–01) and *Amaroecium proliferum* (Maurice et Schulgin, 1884). Since these investigations are incomplete it was thought that the study of an egg with abundant yolk might be of interest in analyzing the extent to which yolk modifies the processes of mosaic development. *Amaroecium constellatum* was chosen because of the apparent twisting of symmetry in its axial structures, the neural tube lying to the left of the notochord rather than dorsal to it.[1]

Material and Methods

Amaroecium constellatum is a compound Ascidian commonly known along the eastern shore of the United States as "sea pork." The zooids are elongate and clustered together to form thick fleshy colonies. The gonads are located in the long post-abdomen, testes posterior to the ovaries which crowd up against the lower part of the abdomen. Fertilization is internal and the embryos develop within "brood spaces." The embryos are located along the length of the ascidiozooid according to the degree of development, the eggs and early stages being lodged in the post-abdomen and lower abdomen; the later stages of tadpoles, in the thoracic region from which they escape when development is completed. The shape of the eggs varies by crowding from spherical to polyhedral. The average diameter of the fixed egg is 250 micra.

The breeding season extends throughout the summer months. All stages are abundant during July and August. During the latter part of June and the early part of September the embryos are few in number. Eggs and embryos are obtained by squeezing the adult colonies in a finger bowl of sea water. Some of the earlier stages may be obtained by dissecting the individual members of colonies under a low power microscope. The post-gastrulation stages will continue to develop in tanks in the laboratory but the pre-gastrulation stages are extremely sensitive. They disintegrate shortly after their removal from the adults without completing the divisions then in progress. These embryos must be used as soon as removed and when the material is fresh.

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[1] I express deep gratitude to Professor E. G. Conklin for his interest and encouragement in the preparation of this paper.

The study of whole embryos is made difficult but not impossible by the character and arrangement of the test cells. Observations, as far as the establishment of the neural plate, were made on whole specimens. They were fixed in Bouin's fluid and preserved in 70 per cent alcohol in which they were mounted on shallow depression slides in vaseline cells to permit rolling them about for examination. The picric acid is retained strongly by the yolk granules whereas the yolk-free cytoplasm, immediately about the nucleus, is colorless. The contrast provides a reliable means for the identification of cells. The position of the spindles can be ascertained and the orientation of the cells known with certainty. Both reflected and transmitted light were used.

Berrill's (1932) technique of hydrolyzing the test with digestive juices was tried but by the time the closely applied test was removable the enzymes had attacked the cells themselves.

The critical stages immediately preceding and following gastrulation were drawn with the aid of a camera lucida, then embedded and sectioned in the manner suggested by Doctor Eleanor Slifer (oral communication) for yolk-laden eggs. All reconstructions were made from serial sections studied in conjunction with whole cleared specimens.

This study deals with the embryo as far as the end of gastrulation. A second paper is concerned with organogenesis in the tadpole.

Early Development

Since there are many maturation spindles in evidence in the unfertilized eggs but no polar bodies formed, it seems reasonable to conclude that Amaroecium agrees with Styela in extruding its first polar body at fertilization. The egg at this time is plentifully supplied with yolk granules. The test cells are embedded in the peripheral cytoplasm in a compact layer and the follicle cells are tightly pressed against the chorion. After fertilization the test cells are clustered outside the egg and inside the chorion in small irregular groups, some remaining embedded in the cortical cytoplasm. The chorion is lifted from the surface leaving a wide perivitelline space. As cleavage proceeds the test cells completely fill up this space as well as any available cleavage furrows and depressions on the surface of the young embryo. On its external surface the test presents the appearance of a pavement epithelium. In section it consists of compact layers of spindle shaped cells with deeply staining nuclei.

The first cleavage is meridional and divides the egg into two blastomeres which represent the right and left halves of the future embryo. The right blastomere is slightly smaller than the left one (Fig. 1, C; 2, B). In some cases the disparity seems greater than in others, due, probably, to variations in shape of the egg resulting from pressure of contiguous eggs. It has been shown in the simple Tunicates and in Amphioxus (Conklin, 1905, 1932) that the cleavage nucleus lies posterior to the center of the egg but in the midline. The disparity in size of the first two blastomeres in Amaroecium indicates that the meeting of male and female pronuclei is effected not only in the posterior region of the egg but also to the right side of the midline (Fig. 1, B; 2, A). In Amaroecium, therefore, there is a double eccentricity of the zygote nucleus, posterior and to the right of the main axis of the egg.

In all the sections of Amaroecium eggs examined the polar bodies lies eccentrically with respect to the main mass of yolk (Fig. 1, A). By assuming that the germinal vesicle always lies to the right of the apex of the elliptical egg and also that the sperm penetrates on the same side it is possible to explain the lateral eccentricity of the zygote nucleus to the right of the median axis; this, in turn, explains the constant inequality in size of the first two blastomeres.

The second division also is meridional and at right angles to the first, thus dividing the egg into two anterior and two posterior blastomeres. The posterior blastomeres are smaller than the anterior two. Beginning with the smallest of the four they fall into this order: the right posterior, the right anterior, the left posterior and the left anterior. The nuclei of all lie at the extreme tip of the cells toward the animal pole (Fig. 2, C, D). The third cleavage cleaves the egg in the latitudinal plane into four micromeres at the animal pole and four heavily yolked macromeres at the vegetative pole.

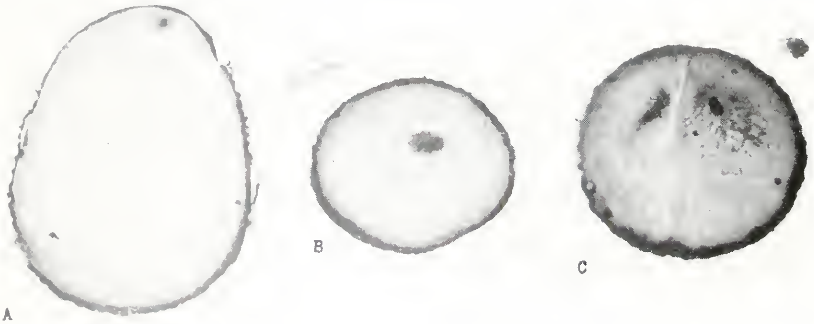

FIGURE 1. Photomicrographs of A. Fertilized egg; first polar body extruded, spindle for second polar body forming. Chorion is broken at the animal pole. Test cells bulge through the chorion. B. Cross section through animal pole of egg after fusion of the pronuclei showing double eccentricity of the nucleus posterior to and lateral to the midplane of the egg. C. Two cell stage; smaller cell is right blastomere. Magnification—250 ×.

The nuclei and their areas of yolk-free cytoplasm elongate in the direction of the next division and the spindle remnants remain clearly evident after the division is completed. These two features constitute valuable means of identifying the axes of the embryo.

The spindles of the two anterior micromeres are parallel with the antero-posterior axis whereas in the two posterior micromeres they are transverse to this axis and the posterior cells form an arc around the anterior cells (Fig. 3). The elongate cytoplasmic areas of the posterior macromeres in preparation for their next division converge towards the animal pole. The cytoplasmic areas of the anterior macromeres lie parallel with the lateral borders of the overlying micromeres (Fig. 3).

It will be convenient for purposes of ready reference to designate the cells by the letters used by Conklin in his study of cell lineage in the Tunicates. By underlining the cell designation of the left side he distinguishes them from their corresponding members on the right side. In the eight cell stage the anterior left macromere is $\underline{A}$ 4.1, the right one is A 4.1. The corresponding micromeres are

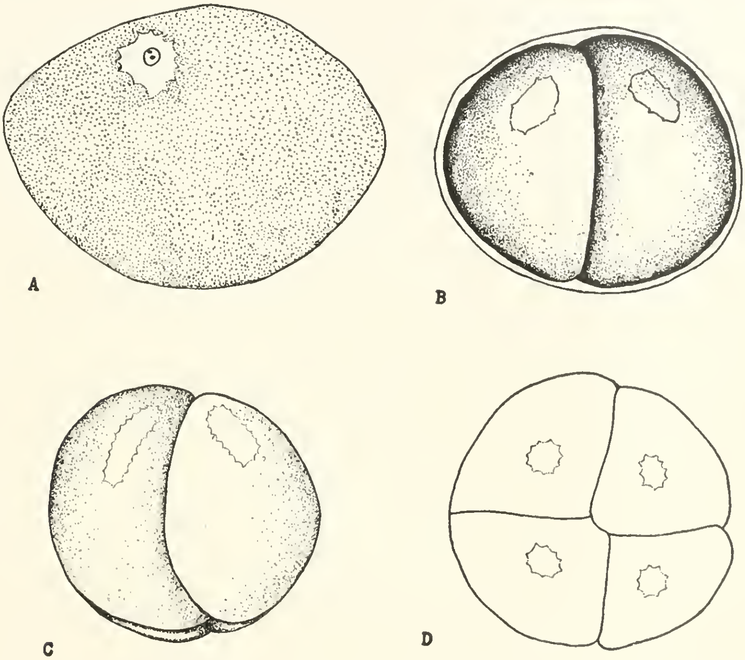

FIGURE 2. A. Fertilized egg before first division, B. Two cell stage; posterior view, C. Four cell stage; anterior view, D. Polar view of four cell stage. Vegetative pole position of nuclei showing. Except where noted all magnifications are 266 ×.

$\underline{a}$ 4.2 and a 4.2. The posterior left and right macromeres are $\underline{B}$ 4.1 and B 4.1 respectively. Their corresponding micromeres are $\underline{b}$ 4.2 and b 4.2 (Fig. 3).

The fourth division is significant in that it accomplishes the distribution of cytoplasmic substances to areas similar to those recognizable in forms with less yolk in their eggs. All the micromeres divide at approximately the same time. Both pairs of macromeres divide unequally into a smaller pair of cells toward the animal pole and a large pair of yolk-charged macromeres occupying the entire vegetative hemisphere. The median derivatives of the anterior pair constitute the chorda-neural crescent, the posterior derivatives are combined mesoderm and ectoderm. All sixteen cells can be seen in polar view (Fig. 4).

Since the cleavages are not synchronous beyond the sixteen cell stage it may be well at this point to summarize the cell lineage in the egg of Amaroecium. The micromeres, designated by small letters in the figures, produce the ectodermal cells that eventually grow over all the other cells of the embryo. The "A" macromeres give rise to the chorda-neural crescent, $\underline{A}$ 5.2 and A 5.2, and half of the endodermal quadrant, $\underline{A}$ 5.1 and A 5.1. The "B" macromeres give rise to the mesodermal crescent, $\underline{B}$ 5.2 and B 5.2, and the other half of the endodermal quadrant, $\underline{B}$ 5.1 and B 5.1 (Fig. 4).

The pattern corresponds exactly to the pattern of Ascidian mosaic development in Styela. The generous provision of yolk in the egg of Amaroecium prevents the appearance of a blastocoele cavity and disposes the presumptive mesodermal cells to a position farther towards the animal pole than this crescent of cells occupies in the egg of Styela where the presumptive endoderm cells are much smaller in size. The hereditary pattern of development is not disturbed by the accumulation of yolk. The mechanics of the process are altered but the same relative positions of the presumptive embryonic areas are assumed. The embryonic areas consist of an endodermal quadrant of large macromeres on the dorsal side, i.e., toward the vegetative pole, with a chorda-neural crescent of two cells lying anterior to them and a mesodermal crescent of two cells lying posterior to them. The ectodermal micromeres occupy the entire animal pole or future ventral side of the embryo. All the cells on the right side are smaller than their sister cells on the left side. Otherwise the cells are disposed symmetrically with respect to the median plane of the embryo.

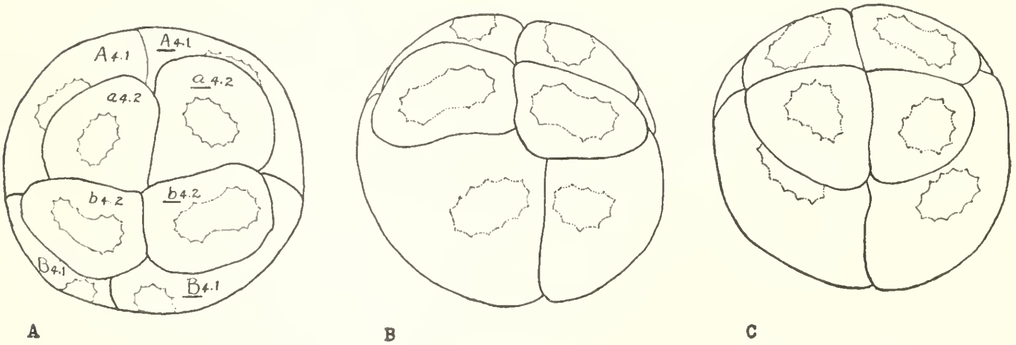

FIGURE 3. Eight cell stage; direction of spindles indicated by stippled areas, A. Animal pole, B. Posterior view, C. Anterior view.

Before the fifth cleavage the micromeres shift in position; the "a" cells spreading transversely, b 5.4 adjacent to them and b 5.3 overlapping the mesodermal cells (Fig. 5, A). The embryo passes through a twenty-two cell stage in its fifth cleavage. The macromeres and their derivatives divide first, those on the right side preceding those on the left. The mesodermal cells divide meridionally, increasing the number of cells in that arc to four. The posterior macromeres also divide meridionally but unequally, producing two smaller lateral mesodermal cells towards the animal pole and two median elongate macromeres (Fig. 5, C, E). Each of the anterior pair of endodermal macromeres divides into two unequal cells, a smaller one on each side of the chorda-neural crescent and a larger median one (Fig. 5, D).

The four large macromeres meet in a median furrow at the vegetative pole. The chorda-neural cells divide later into a transverse row of four cells.

In the twenty-two cell stage there are three mesodermal cells on the right side, B 6.2, B 6.3, B 6.4 and their corresponding cells on the left (Fig. 5, 6). When they divide at the sixth cleavage the dorsal derivative of B 6.2 which is B 7.4 and of B 6.4 which is B 7.8 are presumptive muscle cells. The ventral members and both derivatives of B 6.3 are mesenchyme. Reference to Figure 5 will show that B 6.3 lies at the mid-region of the posterior lip of the blastopore. At this division the chorda-neural cells divide into two transverse rows of four neural cells and four chordal cells. The chordal cells lie towards the vegetative pole, the neural cells towards the animal pole and in contact with the ectodermal micromeres (Fig. 7).

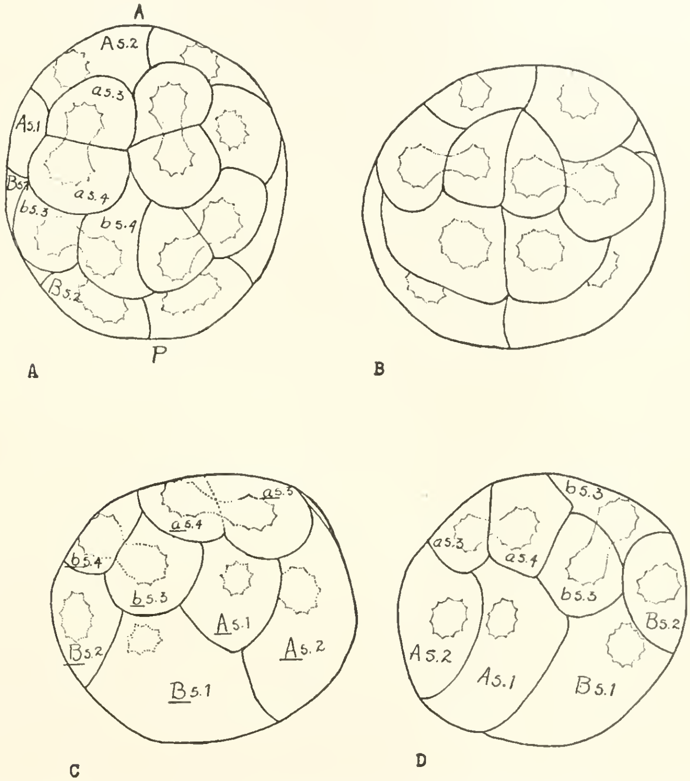

FIGURE 4. Sixteen cell stage, A. Animal pole, cells on right side designated, B. Posterior view, C. Left side, D. Right side.

No attempt is made beyond this point to follow the lineage of the cells. Since they correspond through the first six cleavages with the cells of Ascidians whose cell lineage can be followed through gastrulation, it may be assumed that their agreement continues through subsequent stages with differences dependent on the mechanics of gastrulation in Amaroecium.

It may be helpful before presenting the process of gastrulation to clarify the terms used in describing it. The blastopore is the margin of cells surrounding the macromeres. Its anterior border consists, at first, of chorda-neural cells and later of neural cells only. It is consistently called the anterior lip (the "dorsal lip" of embryologists dealing with Amphibian forms). Its posterior border ("ventral lip") is the crescent of presumptive mesodermal cells and is called the posterior lip. Lateral regions are referred to as right and left lips respectively.

In Styela, which provides the pattern to be used as a basis for comparison, movements of cells in gastrulation are not modified by accumulation of yolk. The first cells of the mesodermal crescent to be inturned at the lateral lips of the blastopore are mesenchyme cells which come to lie ventrally in the trunk region of the embryo. The cells that converge medially to form the lateral lips after the mesenchyme invaginates are presumptive muscle cells of the tail. The final blastopore is T shaped, neural cells forming the anterior lip, muscle cells forming the lateral margins of the posterior lip. The posterior lip forms the limb of the T where the lateral lips converge toward the mid-plane in a groove. At the posteriormost point in the groove the caudal mesenchyme cells are lodged. Chordal and endodermal cells invaginate, the latter forming a typical archenteron. The blasto-

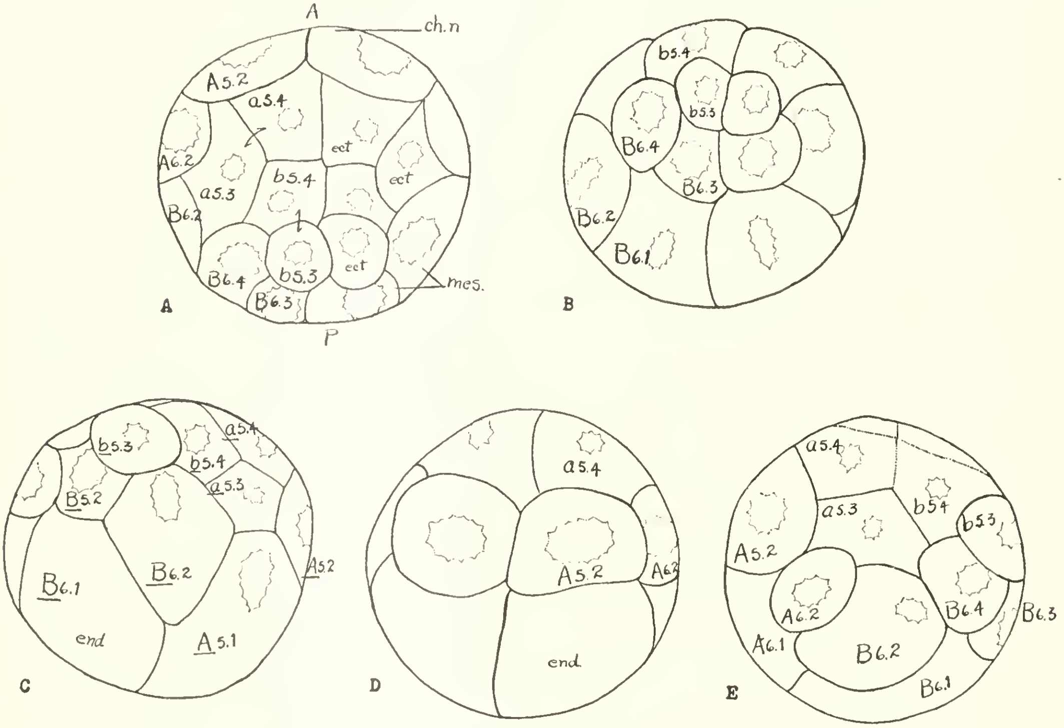

FIGURE 5. Twenty-two cell stage stage.
A. Animal pole; *ch. n.*—chorda-neural crescent; *mes.*—mesodermal crescent; *ect.*—ectodermal micromeres, B. Posterior view, C. Left side, D. Anterior view, E. Right side.

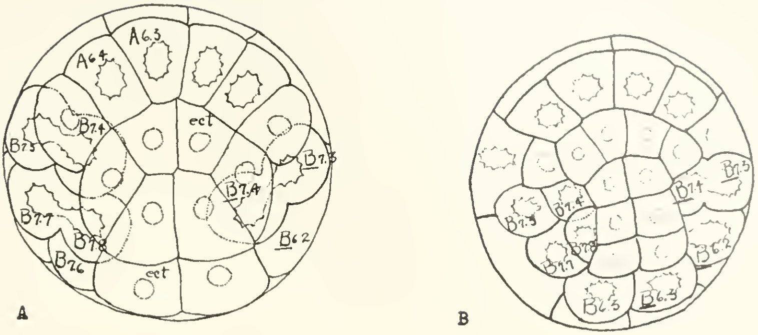

Figure 6. A. Thirty-two to sixty-four cell stage; division on the right side in advance of the left side. B. Same stage, mesodermal cells having completed division on the right side but lagging on the left.

pore closes by posterior growth of the anterior lip and growth toward the mid-line of the lateral lips.

The egg of Amaroecium departs from this pattern of gastrulation in several respects. The margins of its blastopore are established at the sixth cleavage. The anterior lip consists of four chordal cells, the posterior lip of mesodermal cells. Enclosed by the blastoporal lips are the large vegetative macromeres (Fig. 7). In subsequent cleavages they divide into cells of unequal size. The cells at the animal pole are fairly uniform (Fig. 5, 6). All the animal micromeres may be called the epiblast. They differentiate into ectoderm which spreads over the embryo by the process of epiboly.

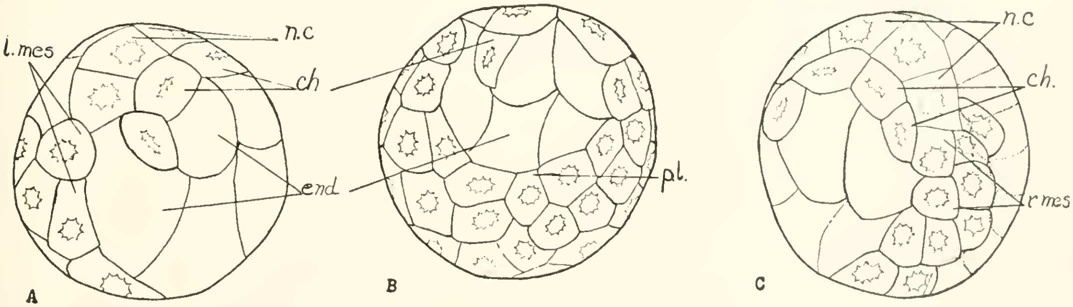

Figure 7. Gastrulation.

A. Viewed from the left lateral blastoporal lip, B. The embryo tilted toward the animal pole to show the complete lip of the blastopore. The angle distorts the size of the endodermal area, C. Viewed from the right blastoporal lip; *ch.*—chordal cells; *end.*—endodermal macromeres; *l.mes.*—mesoderm of left lateral lip of blastopore; *n.c.*—neural cells; *r.mes.*—mesoderm of right lip; *p.l.*—posterior lip of blastopore.

The mass of inert yolk in the macromeres prevents an invagination of the potential endodermal cells. These cells, therefore, do not participate in the early movements of gastrulation. Gastrulation commences with activity of the cells in the mesodermal crescent. They divide and move over the surface of the macromeres in the direction of the vegetative pole. The cells on the right side of the crescent precede those of the left side in dividing. Smaller size of the macromeres on the right side and accelerated rate of division of these mesodermal cells effect

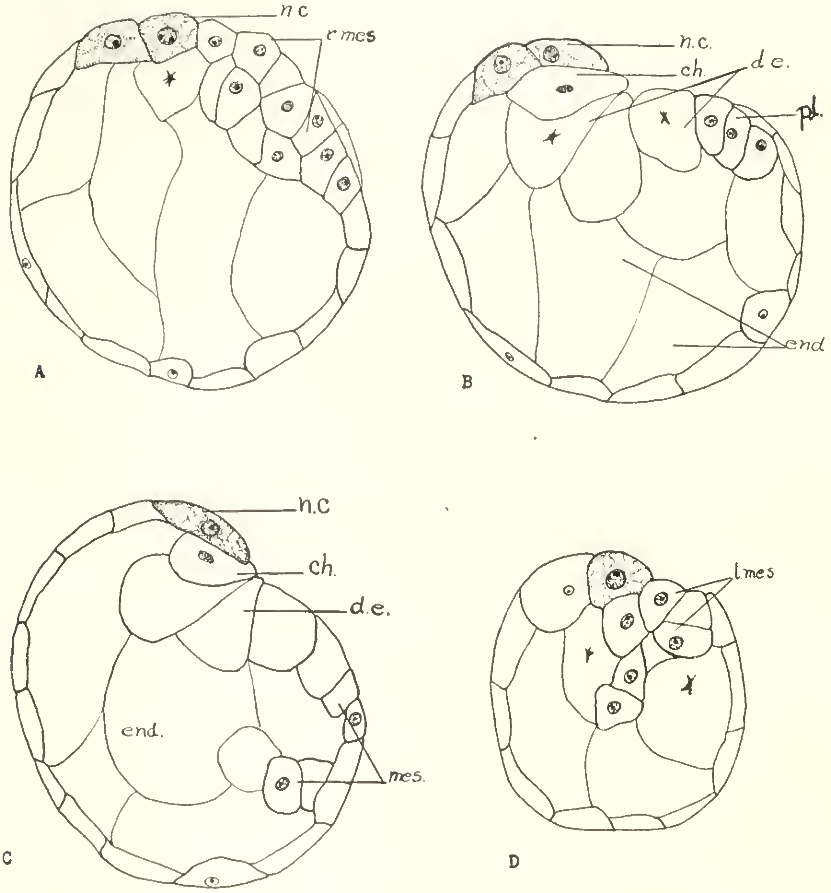

FIGURE 8. Sections through a gastrula during the period of pseudogastrulation.

A. Through the right lip, B. Through the middle of the blastoporal lip, C. Through the region immediately to the left of the preceding, showing the mesodermal cells lower on the left side, D. Through the left lip; depression present where mesoderm meets neural cells; *ch.*—chorda; *d.e.*—definitive endoderm or endoderm of the pharyngeal roof; *end.*—yolk-laden endoderm of floor of pharynx; *mes.*—mesoderm.

a change in shape of the blastopore from circular to an irregular oval. More rapid overgrowth of the right lateral region of the posterior margin results in a narrowing of the blastoporal rim on that side in the antero-posterior direction (Fig. 7). The cells converge medially to form lateral lips as they do in Styela but they converge more rapidly from right to left and thus distort the shape of the blastopore. The right lateral margin defines more of a horizontal curve than the left (Fig. 9, A, B). The cells of both chordal and neural crescents, the anterior lip, increase in number to eight.

As the blastopore becomes smaller changes occur in the endodermal area. The macromeres divide into a number of polyhedral cells, those at the surface of the region enclosed by the blastopore being smaller than those constituting the internal yolk mass (Fig. 8, B, C). This layer of cells may be called the endodermal plate. Corresponding in shape with the blastopore it is a small oval region tapering to a narrow point on the anterior right side where the blastoporal lips are approaching each other more rapidly than they are on the left side. The cells of the endodermal plate change in shape from polyhedral to pyramidal, their apices tapering into a cleft-like depression on the surface formed simultaneously with their change in shape. Their broad bases rest on the larger endodermal cells in the interior of the embryo (Fig. 8, B, C). Such an invagination may be called a "pseudo-invagination" since it closes again without the formation of an archenteron.

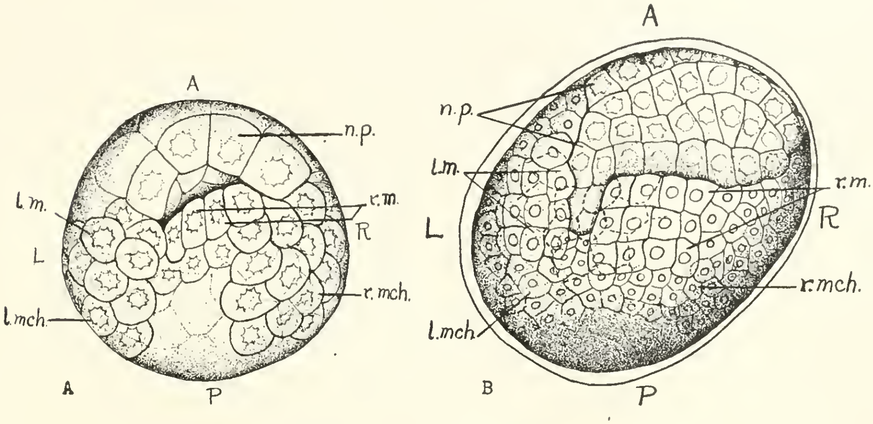

Figure 9. Late gastrulation.

A. Dorsal view. Blastopore closed on right side when depression appears in endoderm. Mesoderm growing from right to left in lip of blastopore. B. Posterior view of embryo at the end of the gastrulation period, tilted slightly to show complete left side. Cells with interrupted outlines represent ectoderm, shown only where blastopore has closed; *l.m.*—left muscle cells; *l.mch.*—left mesenchyme; *n.p.*—neural plate; *r.m.*—right muscle cells; *r.mch.*—right mesenchyme.

The chordal cells adjacent to the endodermal plate invaginate with the endodermal cells. They are involuted at the anterior lip of the blastopore and come to lie immediately underneath the neural plate and dorsal to the endoderm (Fig, 8, A, B, C). With involution of the chordal cells the blastopore closes on the right side. The mesodermal cells of the posterior margin on this side now occupy their internal embryonic position (Fig. 8 A). The ectodermal cells have overgrown them and the ectodermal cells meet the neural cells at the right side of the neural plate. The remaining mesoderm curves around the region of "pseudo-invagination" to the left end of the neural plate or anterior lip where the mesoderm has been proliferating more slowly. The lateral margins of the posterior blastoporal lip are potential muscle cells of the tail; the central region is caudal mesenchyme.

The relationship of the blastopore regions are the same as they are in gastrulation stages of Styela where the blastopore is finally T shaped. Convergence of the lateral margins toward the median axial plane is asymmetrical and the lateral margins fuse to the left of the mid-line. The blastopore of Amaroecium may be described as an irregular T the right horizontal bar of which is longer than the left (Fig. 9, A, B).

Closing of the blastopore on the right side produces a slight horizontal curve in the neural plate. When the depression in the endodermal plate closes, the cells of the neural plate extend posteriorly. The neural cells slope gradually, the left side lying more posteriorly than the right side (Fig. 10).

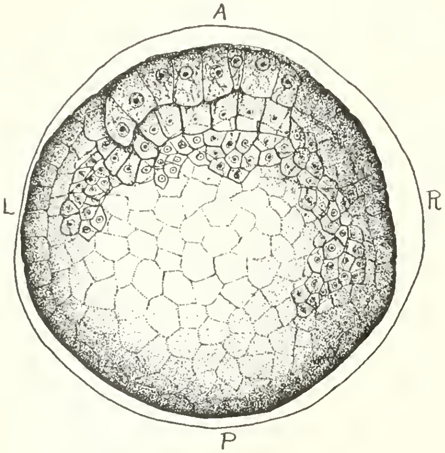

FIGURE 10. Embryo at end of gastrulation. Posterior row of neural cells showing drawn toward mesoderm on left side. Ectoderm in stippled lines; mesenchyme small cells in masses on right and left sides; presumptive muscles stretched across lip and to left of lip.

The activities of all marginal cells in closing the blastopore correspond to the same activities in other Tunicates but the difference in rhythm of division between the cells on the right and the cells on the left deflects the posterior margin of the blastopore to the left (Fig. 8, D). This asymmetrical growth shifts the right muscle cells to the dorsal side of the notochord, the left muscle cells to the ventral side. Convergence and fusion of the cells of the blastoporal margins to the left of the mid-line results in the posterior extension of the neural plate laterally instead of mid-dorsally. The neural tube lies, therefore, to the left of the notochord instead of dorsal to it. Amaroecium is the only Tunicate known in which there is a twisting of the neural tube from a position dorsal to the notochord.

When gastrulation is completed the embryo is approximately spherical with a rudiment of a tail. It lacks an archenteron and the posterior region of the neural plate or potential neural tube is asymmetrical, curving through an angle 90° to the left. Except for these two differences it resembles superficially at this early stage the tadpole of other Ascidians.

Discussion

All the Tunicates whose embryology is known conform to a pattern of mosaic development. *Amaroecium constellatum* despite the fact that its egg is heavily yolk-laden follows the same pattern with modifications contingent upon the mechanical interference of yolk. As Conklin has pointed out, "Cleavage is less constant and fundamental than the type of localization and the two may be relatively independent."

The ooplasmic substances are distributed to cells that assume the same relationships found in forms not filled with yolk. The main mass of inert yolk remains at the vegetative pole and spreads the early blastoporal lips into a circle wider than that of Styela or Amphioxus although the cells are disposed in the same pattern. Ectodermal micromeres occupy the animal pole, yolk occupies the vegetative pole, and between these two areas lie an anterior crescent of mesodermal cells constituting the lip of the blastopore.

Three movements concur in carrying the cells to their final positions where they differentiate into the fundamental structures of the adult body, involution or invagination, epiboly, and convergence or the movement of axial structures into their positions in the median plane. Invagination of the endoderm is impossible but what might be considered an abortive attempt at invagination is made in the depression of the definitive endoderm. It may be called "pseudo-invagination." The depression is accomplished by a change in shape of the endodermal cells whereby they are depressed below the surface. It is not an invagination of cells into a segmentation cavity. Neither does it effect the formation of an open archenteron.

As the mesoderm proliferates over the surface of the endoderm the epiblast cells grow over them. By the process of epiboly, therefore, both mesoderm and endoderm are established in their typical relationships. Since the mesoderm continues to spread between ectoderm and endoderm its movement may be regarded as invagination.

Convergence is the process most violently disturbed by the modified pattern of mosaic development. Its disturbance is due primarily to the bilateral inequality in the size of the first two blastomeres which is responsible for difference in rhythm of division between the right and left halves of the embryo. Cell movements of gastrulation, being greater on the right side than on the left, the blastopore becomes asymmetrical in shape. Cells are thus prevented from converging towards the mid-plane. The lateral margins of the posterior border of the blastopore converge and close on the left side and the posterior extension of neural cells curves through a gradual angle to 90° from the mid-dorsal plane.

Dalcq (1938) concludes that the notochord does not induce neural tube formation in the Protochordates as it does in Amphibia (Spemann, 1928). The notochord in Amaroecium is axial and cannot be responsible for the normal asymmetry of the visceral ganglion and neural tube. There is no experimental evidence in the Ascidians to support the dependence of differentiation of the neural tube on presumptive mesodermal tissue. The fact that the asymmetry of the posterior parts of the nervous system in Amaroecium follows the asymmetry of the mesoderm at the blastoporal lip may indicate some degree of dependence between the mesoderm and the differentiation of the nervous system of the trunk in Protochordates.

SUMMARY

1. The egg of Amaroecium contains more yolk than that of any of the other Ascidians whose embryology has been studied.

2. In the two cell stage the right blastomere is always smaller than the left, establishing an inequality in size that persists through subsequent divisions.

3. In the fifth division the cytoplasmic substances are distributed in this fashion: ten ectodermal cells at the animal pole, four endodermal macromeres at the vegetative pole, a crescent of two chorda-neural cells between them on the anterior side, a crescent of six mesodermal cells on the posterior side, two endodermal-mesodermal cells on each side.

4. Decreased size of cells and increased activity in the mesodermal cells on the right side produce asymmetry in the blastoporal lip.

5. Gastrulation is accomplished by the combined processes of overgrowth and invagination.

6. The blastopore closes from right to left, producing a curve in the neural plate through an angle of 90° in the region of the potential neural tube.

7. When the blastopore closes the potential muscle cells lie above and below the notochord, interrupted on the left by the neural tube, on the right by the endodermal rod.

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PELOMYXA CAROLINENSIS (WILSON) OR CHAOS CHAOS (LINNAEUS)?[1]

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INTRODUCTION

Considerable confusion exists concerning the name of the large amoeboid organism which was discovered and named *Pelomyxa carolinensis* by Wilson in 1900. The other name by which this organism is known is *Chaos chaos* (Linnaeus). It is the purpose of this paper to show that the valid scientific name is *Pelomyxa carolinensis* Wilson.

HISTORICAL

The historical data have been presented in full elsewhere (Schaeffer, 1926; Mast and Johnson, 1931) but for clarity of discussion it is necessary to list the pertinent facts.

1. In 1755 Roesel von Rosenhof found an amoeboid organism which he described, figured, and named "der kleine Proteus."
2. In 1758 Linnaeus named Roesel's organism *Volvox chaos* and in 1767 *Chaos chaos* because the name Volvox had been used earlier for the colonial flagellate which today bears that name.
3. In 1900 Wilson discovered a large amoeboid rhizopod in North Carolina which he described and named *Pelomyxa carolinensis*.
4. This organism was again found by Penard in France (1902); Kepner and Edwards, in Virginia (1917); Schaeffer, in Tennessee and New Jersey (1937); and Brandwein, Penn, and Shiel, in New York (1943). It is now being maintained in clone cultures by Schaeffer, Belda, Pace,[2] Rice, and perhaps others.
5. Schaeffer (1926) maintains that Roesel's "der kleine Proteus" and Wilson's *Pelomyxa carolinensis* are identical generically and specifically, and that the valid scientific name is therefore *Chaos chaos* (Linnaeus). Stiles (1905), however, believes that the name *Chaos chaos* (Linnaeus) is the valid name for *Amoeba proteus* Leidy, maintaining that Roesel's "kleine Proteus" is like this common laboratory amoeba.
6. Mast and Johnson (1931) present evidence which shows that Roesel's organism "is neither generically nor specifically like either Leidy's proteus or Wilson's carolinensis." They contend that it is a myxomycete, "an organism usually classified as a plant."

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[1] The author desires to express his grateful appreciation to Dr. T. E. Powell, Dr. C. W. Hagquist, and Mr. T. H. Mackintosh of the Carolina Biological Supply Company for many helpful criticisms in the preparation of this paper.

[2] Dr. D. M. Pace, College of Pharmacy, University of Nebraska, kindly furnished the pelomyxae from which the author's clone was established.

FACTS AND DISCUSSION

1. *It is impossible to ascertain the exact structure of Roesel's "der kleine Proteus."* A careful study of Roesel's figures reveals that these are barely more than outlines containing a mass of dots and circles. One could not by any stretch of the imagination consider these figures sufficient basis for the identification of any amoeboid species. What the dots and circles represent is obscure. Schaeffer (1938) states that Roesel knew nothing about nuclei, contractile vacuoles, crystals, etc., since these had not yet been discovered, nevertheless Schaeffer admits that these structures are quite important for identification.

Stiles (1905) believes Roesel's organism is *A. proteus* (Leidy), its valid name being *Chaos diffluens* (Müller); Schaeffer (1926, 1937, 1938) maintains that it is *P. carolinensis* (W.), its valid name being *Chaos chaos* (L.); and Mast and Johnson (1931) contend that it is neither, finding it to be a myxomycete. This difference of opinion is in itself strong support for the contention that reasonable proof of the identity of this organism cannot be found.

The first description of *P. carolinensis* by which it can be identified was published by Wilson (1900). Schaeffer (1926, 1937) states that the organism he found in Tennessee, and the one he found in New Jersey (1937) and now maintains in clone culture, is identical with *P. carolinensis* (W.) and "der kleine Proteus," holding that it should be called *C. chaos* (L.). However, since it is impossible to ascertain the identity of "der kleine Proteus," the priority rule establishes *Pelomyxa carolinensis* Wilson as the valid scientific name.

2. *A comparison of the characters of P. carolinensis* (W.) *and A. proteus* (L.) *shows that they are generically distinct.*

Schaeffer (1926, 1937) maintains that *A. proteus* (L.) and *P. carolinensis* (W.) are morphologically quite similar, placing them in the genus Chaos. On the basis of serological tests he (1937) suggests that they may be "one and the same species." But he (1916) also finds that *A. proteus* (L.) comprises three distinct species (proteus, discoides, and dubia) which he (1926) later advances to the rank of genera (Chaos, Metachaos, and Polychaos).

This raises two questions. Is there any evidence to show that *A. proteus* (L.) and *P. carolinensis* (W.) are generically identical? Does the evidence justify the creation of three new genera out of the species proteus? The second question has been considered elsewhere (Mast and Johnson, 1931). An answer to the first may be found by comparing the chief characters of the two organisms. Reference to Figure 1, a photomicrograph of the organisms in the same microscopic field, clearly shows a great difference in size. Other differences are not so apparent. These are brought out in Table 1 which summarizes pertinent data from various investigators. Most of the measurements credited to them have been checked by the author without serious disagreement. Nevertheless, it must be borne in mind that all measurements represent averages of numerous determinations on diverse clones.

It will be noted that the two organisms are similar in several respects. Both accomplish locomotion by indeterminate lobopodia and possess bipyramidal crystals, Glanzkörper, and small non-refractile granules. The crystals and Glanzkörper, however, are somewhat larger in *P. carolinensis* (W.) than in *A. proteus* (L.). The table also shows that these organisms differ markedly in size (as

TABLE I

Summarized comparison of Amoeba and Pelomyxa

Character	*Amoeba proteus* (Leidy)	*Pelomyxa carolinensis* (Wilson)
Length	Up to 600 μ (Kudo)	1,500–3,000 μ (Wilson); up to 5,000 μ (Schaeffer)
Diameter	134 μ (Chalkley)	500 μ (Schaeffer)
Volume	0.0024 c. mm. (Chalkley)	0.12–1.20 c. mm. (50 to 500 × 0.0024) (Schaeffer)
Pseudopodia	Locomotion by indeterminate lobopodia	Locomotion by indeterminate lobopodia
Locomotion	Comparatively active; recovers from disturbance quickly	Sluggish; recovers from disturbance slowly
Crystals	Truncate bipyramidal; up to 4.5 μ long (Schaeffer); 1.5 × 2.0 μ (Rice)	Truncate bipyramidal; 1.5 × 2.8 μ; up to 10 μ (Rice)
Glanzkörper	Up to 4 μ (Rice)	Up to 10 μ (Rice)
Smaller granules	About 0.7 μ (Rice)	About 0.8 μ (Rice)
Contractile vacuoles	Typically one at posterior end; systole rapid; new vacuole usually formed at same place; character of permanent organelle (Adolph; Metcalf; Mast); 20–50 μ; av. 35 μ (Rice)	Usually between 5 and 15; as many as 25 or 30; temporary organelle (Belda); systole slow; some gradually disappear (Rice); 30–70 μ; av. 36 μ (Rice)
Nuclei	Typically uninucleate; discoidal; nucleus and granules easily visible in living specimen; av. dimensions 36 × 26 × 18 μ (Rice)	Typically between 300 and 400 nuclei; up to 1,000 (Schaeffer); ovoidal or discoidal (two strains; Schaeffer); nucleus and granules not easily seen. 16–18 μ (Wilson); 20 × 15 μ (Rice)
Food vacuoles	Variable in size and number; several dozen	Variable in size and number; up to 100
Reproduction	Binary fission; nuclear division mitotic	Tripartite division of mother cell following simultaneous mitotic division of nuclei (Schaeffer)

measured by length, diameter, or volume), number and average size of the nuclei, number and character of the contractile vacuoles, and type of reproduction.

These differences are largely those which led Greeff (1874) to establish the genus Pelomyxa. He specifically states that the large number of nuclei forms the principal character of the genus. A comparison of the original descriptions of *P. palustris* Greeff (1874), *P. villosa* Leidy (1879), *P. greeffi* Blochmann (1893), and *P. carolinensis* (W.) shows that they are large, sluggish, naked rhizopods containing numerous nuclei, many vacuoles, and large Glanzkörper.

The genus Pelomyxa, therefore, includes rhizopods with a multinuclear organization, whereas the genus Amoeba, for the most part, those with a uninuclear organization. Greeff (1874), Leidy (1879), Blochmann (1893), and Wilson (1900) recognize this multinuclear organization as sufficient basis on which to establish the genus Pelomyxa. In their contention that *P. carolinensis* (W.) is the valid scientific name for Wilson's organism Mast and Johnson (1931) apparently agree with them. Schaeffer (1926, 1937, 1938) evidently does not accept this point of view.

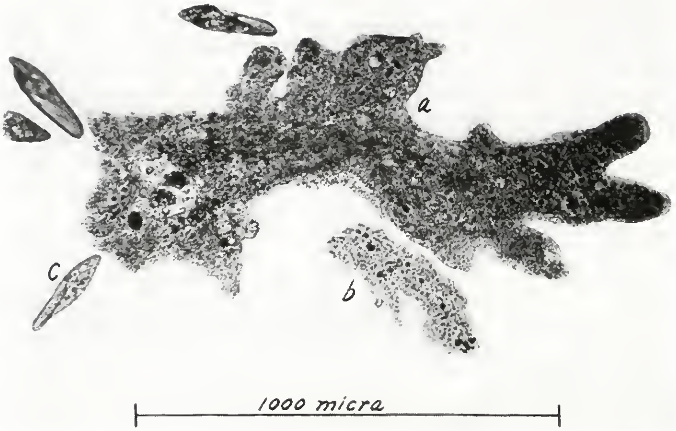

FIGURE 1. Photomicrograph of *Amoeba proteus* (L.) and *Pelomyxa carolinensis* (W.) in the same field. *a*. Pelomyxa; *b*. Amoeba; *c*. Paramecium multimicronucleatum P. et M. (Photograph by Mr. T. H. Mackintosh.)

The evidence presented here shows that *A. proteus* (L.) and *P. carolinensis* (W.) are generically distinct, and that it is illogical, therefore, to place them in the same genus (Chaos).

SUMMARY

1. Since it is impossible to ascertain the exact structure of Roesel's "der kleine Proteus" from his description and figures, it is impossible to identify this organism.

2. *P. carolinensis* (W.) and *A. proteus* (L.) differ in characters of such importance that they must be considered members of different genera.

3. The valid scientific name of Wilson's organism is *Pelomyxa carolinensis* Wilson because he discovered, accurately described, and properly named it.

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A STUDY OF CHROMOSOME ENDS IN SALIVARY GLAND NUCLEI OF DROSOPHILA

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INTRODUCTION

The exact nature of chromosome ends has remained an unsolved problem in the study of chromosome structure. That the end is a specialized part with a characterized behavior is shown by the fact that an intact chromosome end never enters into a permanent association with any other part of the chromsomes and that its absence causes a change in behavior of the chromosome of which it was a part. This is clearly shown by the behavior in maize of broken chromosomes (McClintock, 1938, 1939, 1941, and 1942). Fusion occurs between the raw ends[3] of the two sister halves of the broken chromosomes resulting in a bridge configuration which breaks at anaphase. This "breakage-fusion-bridge cycle" may continue throughout the endosperm tissues, but in the embryo and plant tissues the raw end becomes "healed" and thenceforth no longer shows any tendency toward fusion. In other words, it then behaves as a normal chromosome end regardless of the type of tissue. This shows that there is a difference between a chromosome with a missing end and one with a "healed" end. The nature of the "healing" (or returning to the normal state) is not understood. Additional evidence that chromosomes are dependent for normal behavior upon certain conditions existing at the end is suggested by the paucity of reports of terminal deficiencies. It would be expected that terminal deficiencies would be found with a frequency, the square of the frequency of intercalary deficiencies requiring two breaks. (A small intercalary deficiency may require only one break by an X-ray, Demerec and Fano, 1941.) Nevertheless, terminal deficiencies occur only rarely and are the exception rather than the rule (Kaufmann, 1939a). This would appear to mean either that they are eliminated (Bauer, Demerec, and Kaufmann, 1938), or that there is a tendency for reunion between the two parts of the chromosome. In either case it is true that chromosomes with missing tips are much more rare than chromosomes with an intercalary region missing. There are enough exceptions, however, to show that it is possible for a chromosome to lose its tip and, in some manner, to reform or heal an end which allows the chromosome to function normally (the terminal deficiencies reported by Demerec and Hoover, 1936; and Sutton, 1940). Also terminal inversions (Kaufmann, 1936; Kikkawa, 1937 and 1938), although rare, show the ability of a raw end of the chromosome to heal and assume the role of a normal end. From all of these data we get the impression that the end of a chromosome is not so much a separate entity as the word telomere denotes (for a recent discussion, see Muller, 1941) but rather a very special state which can be achieved by other parts of the chromosome under certain conditions. The separation of bivalents at meiosis presents further evidence that the chromosome end is unique in its behavior. Upon complete terminalization of chiasmata the ends of the separating chromatids frequently hold together with an amazing tenacity (for discussion see Hughes-Schrader, 1943a and b). An association of homologous ends may even be achieved without previous terminalization of chiasmata (Schrader, 1940a and b; Hughes-Schrader, 1943a). Also the bouquet stage strongly indicates a special function of chromosome ends, as pointed out most recently by Hughes-Schrader (1943b) and Schrader (1944).

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[3] The term "raw end" is used throughout to denote the new and unhealed end of a tipless chromosome. It carries no connotation concerning the physical and chemical state of the end and is merely the counterpart of "normal" and "healed."

The purpose of the present research has been to select and analyze some phenomenon characteristic of chromosome ends in the hope that the analysis would lead to a more complete understanding of the nature of that part of the chromosome. The phenomenon selected for study was that of association of non-homologous ends. This phenomenon occurs both in salivary gland nuclei of Diptera and in meiotic nuclei of various forms. The present study is limited to the salivary gland chromosomes of two wild type strains of *D. melanogaster*(Oregon-R and Swedish-b).

In salivary gland preparations the distal ends of any of the five long chromosome arms may be found adhered together, usually two-by-two. This phenomenon of non-homologous association of ends has been noted by several workers and its analysis was undertaken by Hinton and Sparrow (1941) and Hinton and Atwood (1941 and 1942). They found that some of the possible combinations of chromosome ends occurred with a significantly higher frequency than did others. The particular combinations of ends which occur with a non-random frequency varied from strain to strain but were characteristic of any one strain.

The present report adds to these conclusions the results from three lines of investigations: one, the study of the physical nature of terminal adhesions as demonstrated by mechanical manipulation; two, the genetical nature of adhesions, and the factors affecting adhesions; and three, the effect on the adhesion pattern of one strain of subsituting by genetical manipulation a chromosome or chromosome end from another strain. Since each of these points is best dealt with individually, the discussion of the results is presented in connection with the particular data to which it is pertinent. Therefore, the final discussion is of only a general and summarizing nature.

Micromanipulation of Terminal Adhesions

For the purpose of studying the physical nature of terminal adhesions a technique was devised whereby two chromosome arms associated by a terminal adhesion could be pulled apart. This was accomplished with the aid of a Chambers micromanipulator, working with partially fixed glands, as suggested by Buck (1942). The glands were dissected from the larvae freehand under a binocular microscope into a drop of aceto-orcein. At once, before hardening from the fixative resulted, the glands were transferred to a coverslip. A "V" of vaseline on the coverslip prevented the aceto-orcein from flowing away from the glands. The corner of another coverslip was placed over the glands, between the two walls of vaseline, and used to press the glands into a thin layer. The coverslip used to smear the glands was then removed and the coverslip with the smeared gland was inverted and placed on top of a moist chamber on the stage of a microscope above the micromanipulator. The various groups of chromosomes were searched quickly until a terminal adhesion was located. The chromosomes involved were identified and the configuration was drawn, using the 44X objective and 15X oculars. The microneedles were then raised into position and inserted into the chromosomes involved in the adhesion, and pulled apart until the chromosome region between the two needles (including the two adhered ends) broke at some point. This usually involved a marked degree of stretching—from two to three times the original length of the segment. The resulting configuration was then sketched; the figure marked with an ink spot; and the coverslip removed and the tissue further stained with aceto-orcein. The coverslip was subsequently sealed with paraffin face downwards onto a slide. The chromosome configuration was then relocated and the results studied using an oil immersion objective.

Twenty-eight such preparations were successfully completed. In 16 of these, the adhered chromosomes, which were stretched between the microneedles, separated between the two adhered chromosome ends. It should here be emphasized that this did not take the form of a mere separation of temporarily joined chromosome ends, but rather that the chromosome ends were tenaciously held together during the stretching by inter-band-like material which broke only after extreme attenuation. A careful study of the bands on the previously joined chromosome ends failed to reveal a single case in which the ends had gained or lost a band (see Figure 1). The only noticeable effect upon the chromosome ends was, in a few cases, a slight attenuation of the end itself.

Of the remaining 12 preparations, 6 failed to break—the terminal adhesions remained intact even though the chromosomes had been pulled to about three times their original length. After release from the needles, the chromosomes resumed an approximation of their original size and showed no marked effect of the previous stretching. No measurements were made at this point, but the degree of retraction of the chromosomes upon release is probably of the same order as that described by Buck (1942).

Six cases showed intercalary breaks. None of these six breaks were close to the adhered tips, three of them being in the chromocenter and the others in approximately the middle of the chromosome arm (2L in region 28; 3L in 67; and 2R in 52).

Eight successful attempts to disentangle the chromosomes in unbroken nuclei of semi-fixed glands by pulling them free with the microneedles produced three types of configurations. The first type was one in which the chromosomes formed a "star-shaped" configuration with all the bases of the chromosomes joined together and all the tips free. Two such configurations occurred. The second type was a continuous chain. This involved two terminal adhesions (2R-3R; 3L-X) both of which held during the stretching at the expense of the paired bases of the arms.

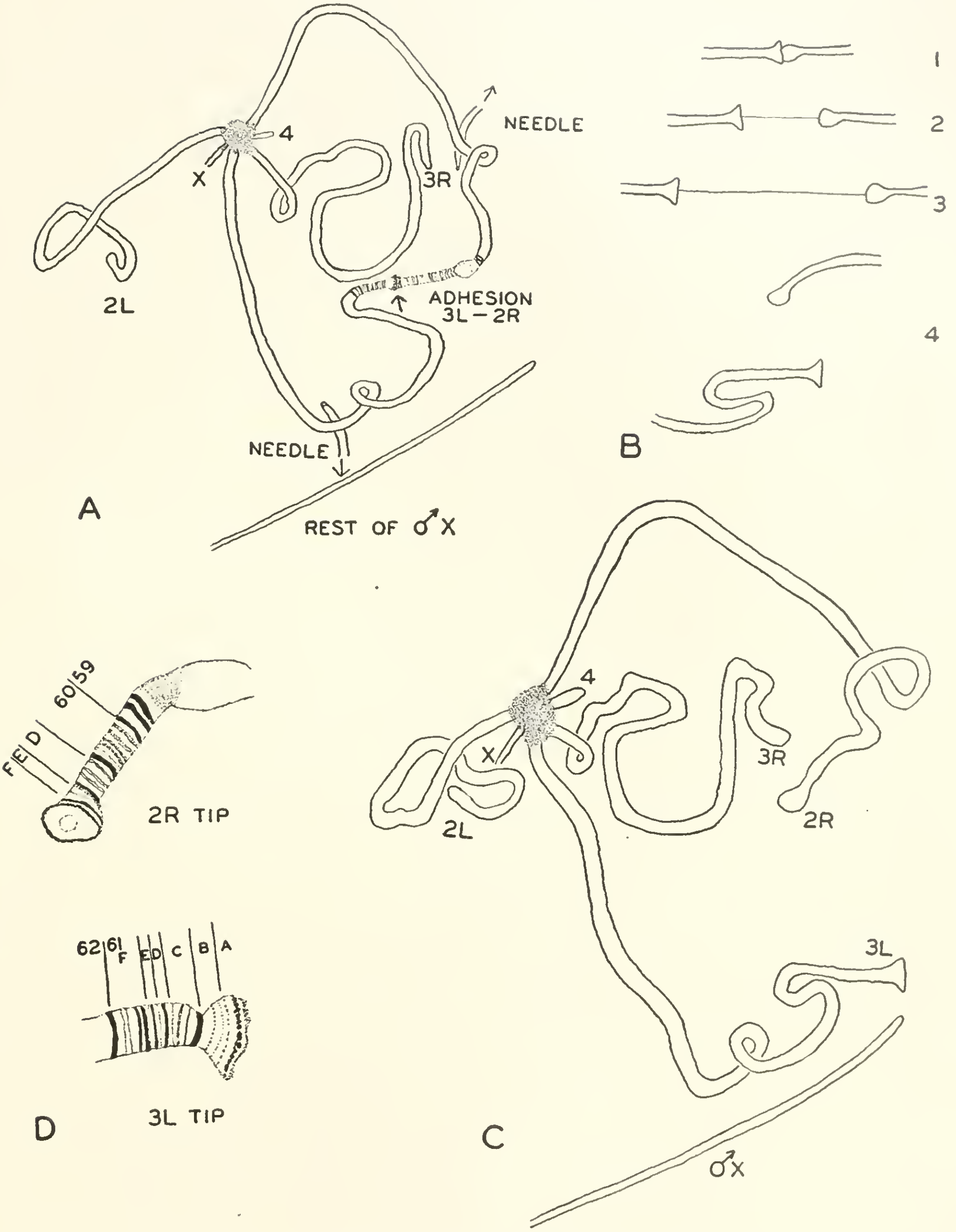
4
NEEDLE
X
3R
2L
ADHESION
3L — 2R
NEEDLE
A
REST OF ♂X
1
2
3
4
B
60|59
F E D
2R TIP
62|61 F E D C B A
3L TIP
D
4
X
3R
2L
2R
3L
C
♂X

FIGURE 1. *A*. Configuration of chromosomes, sketched with needles of micromanipulator in position. *B*. Behavior of the two adhered ends while the needles were being moved apart. 1. Original position. 2. As the streching begins a thread-like material pulls out between the two ends. 3. The maximum extent of the stretching of the material between the two tips. 4. The thread-like material breaks and disappears leaving the ends free and relaxed. *C*. The same confirguration of chromosomes as in *A*, after the ends have broken apart. (Sketched using an oil immersion objective.) *D*. Detailed study of the bands at the ends of the two chromosomes which were previously adherent.

One such case was found. The third involved the chromosomes separating into two groups. This was due to the fact that the bases of the arms were originally joined in two groups—one autosome being alone. Two such cases were noted. Examples of this arrangement of the chromocenter were previously observed and recorded (but not published) when uninjured nuclei in whole mount preparations were being examined for other reasons (Hinton and Atwood, 1941 and 1942). These same arrangements and variations thereof were noted frequently in the broken nuclei. While pulling adhesions apart with the microneedles, the configuration of chromosomes would often be altered, stretching out into any one of the above mentioned types. At least five examples of each type are recorded.

No claim is made that the semi-fixed chromosomes used in these experiments accurately represent the living state. The work on these chromosomes does, however, throw light on such of their physical characteristics as are involved in the preparation of aceto-orcein slides, and shows the behavior of terminal adhesions and the chromosome arrangement from the beginning of fixation in the uninjured nucleus to the time the fully fixed and smeared preparation is observed.

The data show that a terminal adhesion is a condition in which the ends of the two chromosomes are strongly held together by a non-staining material which is continuous with the ends. They also show that the connection between the ends, even though tenacious, is still the weakest place along the chromosomes, with the exception of the adhered bases of the chromosomes. But the very fact that the ends invariably held together as firmly as they did, makes it unlikely that many cases of terminal adhesions are broken apart in the preparation of a slide.

Goldschmidt and Kodani (1942, 1943) noticed "the apparent strength of the cohesion which leads to a breaking off of tips which are attached to another chromosome in chromosome smear preparations." The foregoing data show that when two adhered chromosome ends are mechanically pulled apart, the phenomenon described by Goldschmidt and Kodani does not occur. Instead, it is evident that when a chromosome is fixed to the degree that is usual in preparing a smear, the adhered ends do not break apart with any ease. Even if they do break apart, it does not result in terminal deficiencies and translocations. In addition, if this type of aberration were produced by mechanical means, for every translocation there should be a deficient tip in the same nucleus, but Goldschmidt and Kodani fail to produce such evidence. It is possible that in a more thoroughly fixed chromosome, the phenomenon as postulated by Goldschmidt and Kodani could occur, but as shown elsewhere in the present paper, the frequency of terminal adhesions in a given stock at a given temperature does not vary, regardless of the length of time of fixation. If terminal adhesions break apart in smearing more readily after extreme fixation, then the frequency of terminal adhesions found on smear preparations should vary with the degree of fixation. It seems most unlikely from the data herein described that the small terminal deficiencies and translocations as seen by Goldschmidt and Kodani could be "pseudodeficiencies and translocations produced by mechanical breaks near the chromosome tip following cohesion of the telomere at the tips of two chromosomes." It seems much more likely that the deficiencies and translocations as seen by these authors were true deficiencies and translocations carried in the stocks which they examined; and that due to the small size of these rearrangements they failed to observe them in all nuclei, for it would be extremely difficult to detect consistently an abberation as minute as one involving only one to four bands.

The idea that these small aberrations were carried as characteristics of their stocks does not seem unlikely in light of other work on the subject. Thus Kikkawa (1938) found a number of terminal structural differences between the chromosome ends of various *Drosophila ananassae*. His drawings of these extra bands on the chromosome ends are entirely similar to the drawings presented by Goldschmidt and Kodani (1943) for *Drosophila melanogaster*. Again, Dobzhansky and Dreyfus (1943) found, in Brazilian populations of *Drosophila ananassae*, "variations apparently identical with those described by Kikkawa." Also Bridges made a study of the free ends of the salivary chromosomes of *D. melanogaster* and found various examples of this type of abberation as characteristic of different strains. Unfortunately these data were never published in full but cases are listed in Drosophila Information Service No. 9—Df(2)Ore-R, Df(2)Sw-L, Df(2)Sw-R, Df(3)D^3H^R, Df(3)Mz^L. (See also Bridges and Brehme, 1944, page 59.) There seems, therefore, little doubt that the terminal abberations reported by Goldschmidt and Kodani, Kikkawa, Dobzhansky and Dreyfus, Bridges, and others, are all of the same nature and are inherent in the chromosomes of the individual stock being studied.

That these terminal deficiencies and translocations derive from the separation of terminal adhesions between non-homologous chromosomes, not in salivary gland nuclei, but during meiosis, remains a possibility. It is not inconceivable that a salivary gland nucleus is comparable to a pachytene nucleus of the first meiotic division. If a terminal adhesion occurred during the meiotic prophase and the ends later separated unequally, then the gamete receiving one of the involved chromatids would carry to the offspring a terminal deficiency or translocation. Such meiotic behavior represents one way in which terminal adhesions could play a role in the production of terminal aberrations. Terminal adhesions of non-homologous chromosome ends during the prophase of meiosis have been observed by Slack (1938) in Corixa, Schrader (1941) in *Edessa irrorata*, Ribbands (1941) in Habropogon, Rhoades and McClintock (verbal communication) in *Zea Mays*, and others (see Ribbands, 1941). Also terminal adhesions at meiosis may conceivably be involved with the deposition of matrix substance and the polarization of the chromosomes (see Schrader, 1941).

THE GENETICAL NATURE OF ADHESIONS AND FACTORS INFLUENCING ADHESIONS

Change of frequencies with time

The frequency with which any two chromosome ends were associated was established for the Oregon-R strain in July 1940 (Hinton and Sparrow, 1941), and for the Swedish-b strain in July 1941 (Hinton and Atwood). These percentages are brought together in Table I for the sake of comparison. During the present investigation the frequencies in both Oregon-R and Swedish-b were reexamined (November 1943) by recording the types of adhesions observed from aceto-orcein smear preparations of glands. A marked difference is seen to exist when the current data are compared with those collected in 1940 and 1941. This difference is demonstrated in Table I. The data are from females only and are in per cents of the total cases examined in each case.

TABLE I

Chromosome combinations	1	2	3	4	5	6
	Ore'R 7/40	Ore'R 11/7/43	Ore'R 11/17/43	Sw.-b 7/41	Sw.-b 11/28/43	Sw.-b 11/30/43
X-2L	17.3	21.0	20.0	14.0	3.0	1.0
X-2R	3.1	12.0	4.0	0.9	10.0	14.0
X-3L	17.3	5.0	8.0	16.8	8.0	0.0
X-3R	16.3	20.0	24.0	5.6	9.0	21.0
2L-2R	3.1	2.0	4.0	9.3	1.0	2.0
2L-3L	3.1	1.0	2.0	24.3	3.0	3.0
2L-3R	5.1	1.0	1.0	10.0	1.0	1.0
2R-3L	6.1	1.0	1.0	8.4	5.0	3.0
2R-3R	16.3	31.0	30.0	2.8	52.0	50.0
3L-3R	12.2	6.0	6.0	7.5	8.0	5.0
Total cases	98	100	100	107	100	100

It can be concluded from the data in Table I that:

1. The frequencies within each set of data are non-random. (Chi-square tests give probabilities of much less than .01 in each case that the data are similar to the expected random distributions.)
2. Oregon-R and Swedish-b in 1940–41 differed significantly from each other. (P—less than .01.)
3. Oregon-R, 1943, differs significantly from Swedish-b 1943. (P—less than .01.)
4. Oregon-R, 1940, differs significantly from Oregon-R 1943 (columns 2 and 3). (P—.01 to .02.)
5. Oregon-R, 11/9/43, does not differ significantly from Oregon-R, 11/17/43. (P—.70.)
6. Swedish-b, 1941, differs significantly from Swedish-b, 1943 (columns 5 and 6). (P—less than .01.)
7. Swedish-b, 11/28/43, does not differ significantly from Swedish-b, 11/30/43. (P—.05 to .10.)

The fact that both the Oregon-R and the Swedish-b patterns of adhesions have changed over a three year period of time suggests some mechanism for rapid evolution of the factors involved in terminal adhesions. The possibility exists that the stocks in question are not the identical stocks used in 1940–1941. However, since both Oregon-R and Swedish-b have changed, this seems less likely than if only one stock had changed.

Dominance

It was shown (Hinton and Atwood, 1941 and 1942) that when Swedish-b females were crossed to Oregon-R males, the F_1 females were identical with the Oregon-R parents in the relative frequencies of types of adhesions. This was interpreted to mean that the factors controlling the Oregon-R pattern of adhesions were in some way entirely dominant to the Swedish-b. Since, in the present investigation it has been shown that both the Oregon-R and Swedish-b pattern have changed over a three year period, it was deemed advisable to reexamine the heterozygote (Ore'R/Sw.b). The results are presented in Table II.

The probability that the heterozygote has an adhesion pattern resembling that of the Swedish-b chromosomes is .10 as shown by an χ^2 test. However, the probability that the heterozygote shows the same pattern as the Oregon-R parent is much greater (P—.50); yet, at the same time, an equal probability (.50) is shown that the heterozygote resembles the intermediate between the two parental patterns. Therefore it seems impossible at this time to conclude whether dominance has been maintained by Oregon-R or whether the dominance has been lost and Oregon-R and Swedish-b now have an equal ability to determine the adhesion pattern of the F_1, and either modify or compensate each other. However, if an examination is made of the only two classes wherein Oregon-R and Swedish-b are really dissimilar (X–2L and 2R–3R), then an χ^2 test shows the following: the probability that Ore'R and Ore'R/Sw.-b are similar—.10; that Sw.-b and Ore'R/Sw.-b are similar—less than .01; and that Ore'R/Sw.-b and the theoretical intermediate between Ore'R and Sw.-b are similar—.70. It would appear from this analysis that Oregon-R has lost its dominance completely and that the heterozygote is merely the intermediate between the two parental strains in its adhesion pattern.

TABLE II

Females, 1943

Chromosome combinations	Per cent adhesions Ore'R	Per cent adhesions Ore'R/Sw.-b	Per cent adhesions Sw.-b
X-2L	20.5	12.5	2.0
X-2R	8.0	15.5	12.0
X-3L	6.5	6.5	4.0
X-3R	22.0	16.0	15.0
2L-2R	3.0	3.0	1.5
2L-3L	1.5	2.0	3.0
2L-3R	1.0	2.0	1.0
2R-3L	1.0	1.0	4.0
2R-3R	30.5	39.0	51.0
3L-3R	6.0	2.5	6.5
Total cases	200	200	200

Cytoplasmic effect

In order to observe the pattern of adhesions of identical chromosomes in different cytoplasms, the reciprocal crosses between Oregon-R and Swedish-b were made. Swedish-b females crossed to Oregon-R males produce F_1 daughters heterozygous for a complete set of Oregon-R chromosomes and a set of Swedish-b chromosomes, in Swedish-b cytoplasm (Ore'R/Sw.-b). Swedish-b males crossed to Oregon-R females give identical results with the exception that the cytoplasm is Oregon-R. The data from these two types of heterozygous daughters are presented in Table III.

The data show that the cytoplasm probably has no effect on the frequency of types of adhesions. An χ^2 test gives a probability of .10 that the two sets of data are similar. But here again, if the two crucial classes (X–2L and 2R–3R) are examined, they are exactly similar in the two sets of data. Therefore the factors which may be responsible for the specificities shown in terminal adhesions appear to be characteristic of the chomosomes and not dependent upon the genotype of the cytoplasm. This is in agreement with the conclusion reached by Hinton and Atwood (1941).

Temperature effect

A preliminary experiment indicated that temperature has an effect on the total incidence of terminal adhesions. By counting the number of nuclei examined and the number of terminal adhesions found, it was discovered that if the culture bottles remained at 17° during the development of the larvae approximately 20 per cent of the nuclei contained a terminal adhesion; if the culture bottle remained at 23°, only 13 per cent of the nuclei contained an adhesion. In order to verify this conclusion the experiment was repeated with an attempt to collect more exact data. Females were allowed to lay eggs for 24 hours in culture bottles placed at 17°, and the flies then discarded. A second set of culture bottles received the same treatment at 23°. The culture bottles remained at their respective temperatures until the larvae were ready to pupate, at which time salivary gland preparations were obtained. Fifty figures were examined and the per cent of figures containing adhesions was calculated. Five such sets of data were collected from the cultures at 17°, and three from the cultures at 23°. The average percentage of adhesions per 50 nuclei occurring at 17° differs significantly from that at 23°. At 17° the mean percentage was 20 with a variation of ± 6 per cent (14–26 per cent); at 23°, 13 ± 4 (9–17 per cent). The conclusion seems justified that the frequency of terminal adhesions varies with temperature.

TABLE III

Chromosome combinations	Per cent adhesions Ore'R/Sw.-b in Ore'R cytoplasm	Per cent adhesions Ore'R/Sw.-b in Sw.-b cytoplasm
X-2L	13	12
X-2R	18	13
X-3L	5	8
X-3R	19	13
2L-2R	1	5
2L-3L	0	4
2L-3R	3	1
2R-3L	1	1
2R-3R	39	39
3L-3R	1	4
Total cases	100	100

To determine during which period of larval development temperature has its effect, culture bottles were placed at 23° for 24, 48, 72, and 96 hours respectively, and then removed to 17° where they remained until the larvae were ready to pupate. It can be seen from Figure 2 that there is little effect from the temperature as long as the larvae spend no more than the first three days of their development at 23°. After that the percentage of adhesions rapidly decreases. There are two ways to interpret this decrease in the frequency of adhesions: either terminal adhesions are sensitive to temperature during the fourth day of larval development; or there is an accumulative effect of temperature requiring at least three days. Further experimentation will make it possible to select between these two possibilities, but at this time only these preliminary data on temperature effect have been completed.

It was suspected that some cytological phenomenon could be correlated with the temperature effect. Salivary gland preparations were obtained from second instar larvae (the stage reached at the end of the third day at 23°). This preliminary cytological investigation failed to reveal a clear picture of the chromosomes since they are most difficult to interpret at this early stage. It will be necessary to employ various cytological techniques before the question can be entirely settled. The results of the preliminary investigation do not exclude the possibility that terminal adhesions have not formed at the time of the second instar.

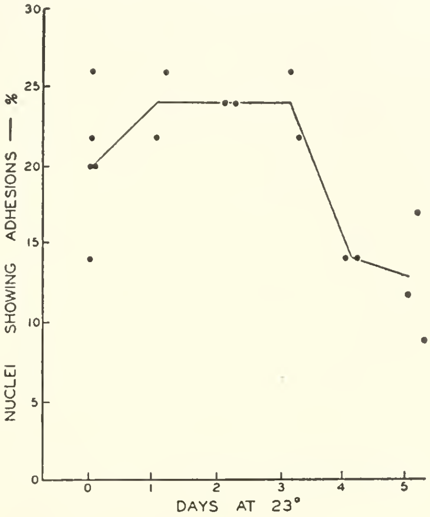

Figure 2.

It will be noted, Figure 2, that the frequency of adhesions is slightly greater when the larvae are placed at 23° for the first several days and then returned to 17° than when the larvae undergo their entire development at 17°. If this is a significant difference, and only more detailed analysis will clarify that, then an explanation becomes difficult. It would indicate that there may be two occasions during the development of the chromosomes when temperature can effect terminal adhesion formation, or that there may be two different ways by which temperature exerts its effect.

In order to locate 100 terminal adhesions in the Swedish-b strain in cultures raised at 17° it was necessary to study, on the average, 45 smear preparations of pairs of salivary glands; but in order to locate 100 adhesions in the same strain raised at 23°, it was necessary to study 128 smear preparations. Yet, when the 100 adhesions from 45 smear preparations are compared as to types of adhesions with the 100 taken from 128 preparations, it is apparent that the terminal adhesion pattern (specificities) remains unaltered. This is demonstrated by the data in Table IV. An χ^2 test gives the following probabilities that these sets of data are similar: Column A compared to column C – P = .80; A to D = .80; A. to E = .95; B to C = .60; B to D = .05; B to E = .02; columns A plus B compared to C – P = .99; AB to D = .70; AB to E = .50; and columns A plus B compared to columns C plus D plus E – P = .70. Therefore, it can be concluded that temperature does not affect the types of adhesions which occur, but only the total incidence of adhesions.

TABLE IV

Chromosome combinations	Per cent adhesions Sw.-b 17°		Per cent adhesions Sw.-b 23°		
	A	*B*	*C*	*D*	*E*
X-2L	3	1	3	3	2
X-2R	10	14	14	7	8
X-3L	8	0	5	8	10
X-3R	9	21	17	13	8
2L-2R	1	2	2	3	3
2L-3L	3	3	2	2	2
2L-3R	1	1	1	4	3
2R-3L	5	3	5	5	7
2R-3R	52	50	46	46	48
3L-3R	8	5	5	9	9
Total cases	100	100	100	100	100

Intercalary adhesions

The ends of the chromosomes not only adhere to each other, but are also found adhered to regions along the length of the chromosome arms or to the bases of arms. Preliminary data on intercalary adhesions were reported by Hinton and Atwood (1941). Since that time 180 cases have been recorded which represent sufficient data to justify an analysis. All of these data were used to plot the frequency with which the various chromosome regions were involved in adhesions and this was compared with values expected on a random basis (number of adhesions divided by the number of chromosome regions as shown on Bridges map—100). A striking deviation from randomness was apparent, with some regions containing as high as 12 per cent of the total cases found, while in other places along the chromosomes as many as five consecutive regions contained no adhesions. In order to treat the data statistically in the most simple manner, each of the five long chromosome arms was divided into four parts by using every five regions on the Bridges map as a unit. These 20 sections were then compared with the theoretical random distribution. An χ^2 test gave a probability of less than .01 that the adhesions could be distributed along the chromosomes at random. The conclusion is justified that there are certain regions of the chromosomes which are associated in adhesions with a high frequency, while the majority of regions show the expected frequency.

The more distal regions of the chromosome arms show a higher frequency of adhesions than do the more proximal. This is best illustrated by Figure 3 in which are shown the five chromosome arms divided into four regions and plotted against the percentage of adhesions occurring per region. The data pictured in Figure 3 involve only intercalary regions, the data for the tips adhered to these regions being reserved for separate analysis.

Since the distal fourth of each chromosome arm shows a greater frequency of adhesions, it is necessary to assume, either that any material responsible for this type of pairing is present in greater abundance near the ends of the chromosome, or that due to the arrangement of the chromosomes, the distal regions are more accessible to the tips of other chromosomes. If the first hypothesis were true and it be postulated that this type of pairing is due to the attraction of heterochromatin, than a greater amount of intercalary heterochromatin should be located in the distal quarter of each chromosome arm than in any other region. There is no cytological evidence to support such an assumption, but since some heterochromatin regions present in dividing chromosomes do not appear in salivary gland chromosomes (Hinton, 1942) such a possibility is not eliminated. If the second hypothesis were true then the differential frequency would be due solely to mechanical causes.

Those regions which have a strikingly high occurrence of adhesions are shown in the following list:

Chromosome arm	Regions
X	2B, 3C
2L	21/22
2R	56, 58
3L	—
3R	99/100, 100C/D

Sixty-six additional places along the length of the chromosome were found to be involved in adhesions, but none of these with a surprisingly high frequency. Kaufmann (1939b) studied the position of X-ray induced breaks along the X chromosome and discovered at least 12 regions with a breakage frequency considerably higher than the expected frequency of a random distribution. He suggested that these regions contain heterochromatin which would account for the high breakage by X-rays and for the fact that these regions occasionally pair with each other and with the chromocenter. The question arises: Are the regions which show a high frequency of adhesions correlated with those showing a high breakage frequency? Table V, shown on the next page, makes such a comparison.

It is true that some regions with a high breakage frequency are found to be involved in adhesions, but on the whole neither a positive nor a negative correlation exists. Since those regions which have the highest adhesion frequency are not the same regions which show a high breakage frequency, non-specific heterochromatin certainly cannot be the important factor in both cases, although the possibility is not eliminated that it is important in one case or the other. Such a comparison as this is justified since the data indicate that neither the breakage frequency (Kaufmann, 1939b) nor the adhesion frequency (see following paragraph) varies for a given region in various strains.

Since it had been demonstrated that the pattern of end-to-end adhesions is different in different strains, the question arises as to whether the adhesion involving intercalary regions also express different specificities in different strains. It is difficult to answer this question definitely due to the relative paucity of data as compared to the several thousand places along the chromosomes that adhesions might occur, but in the Oregon-R strain 69 cases of intercalary adhesions have been recorded; and 46 in the Swedish-b strain, and when these cases are plotted against chromosome regions and the two sets compared it can be observed that the regions showing high frequency of adhesions are usually the same in each case and that the distribution is in general very similar. At least from the data on hand the indications are that even though intercalary adhesions are not at random, the specificity remains constant from strain to strain.

Table V

Regions found by Kaufmann to have a high breakage frequency	Regions found by Hinton to be involved in adhesions	
	Region	Number of adhesions
	1C/D	1
1F		
	2B	11
	2D	1
	3B	1
	3C	22
	3D	1
	4B	2
4E		
	5	3
	5/6	2
7B	7B	1
7C		
8B		
	8F	1
	8/9	1
	9	2
	10A	1
11A	11A	1
12D	12D	2
12E		
	14	1
	15/16	1
16E	16E	1
16F		
	18E/F	1
19E	19E	2
19F		
	20B	1

So far this discussion of intercalary adhesions has dealt only with the subterminal regions involved in the adhesion with no regard to which chromosome ends were involved. Data are presented in Table VI showing the number of times each chromosome end is involved in an intercalary adhesion (in per cent of the number of cases observed).

From these data a difference in the behavior of the ends in the two strains is apparent. An χ^2 test gives a probability of less than .01 that the two sets of data are similar. It therefore appears that the intercalary regions involved in adhesions remain the same from one strain to another but the chromosome ends involved vary from one strain to another. Another point of interest in these data is the correlation between the behavior of chromosome ends involved in intercalary adhesions and those involved in terminal adhesions. It may be recalled that in Swedish-b, 2R and 3R adhere together with a frequency much greater than they do in Oregon-R. In Table VI it can be seen that both 2R and 3R are involved in intercalary adhesions with a frequency higher in Swedish-b than in Oregon-R. The same correlation exists for 2L and 3R. The only chromosome end which behaves the same in terminal adhesions in both strains is 3L, and it is found also to be involved in intercalary adhesions with about equal frequency in both strains (8 per cent and 6 per cent). A correlation is indicated between the potentialities of a chromosome end participating in terminal adhesions and in intercalary adhesions.

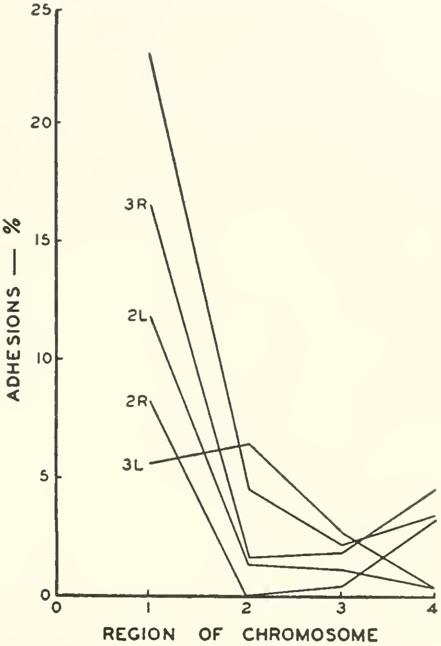

FIGURE 3. The number of intercalary adhesions is plotted against chromosome regions. Region 1 is the most distal in each case, but does not include the tip of the chromosome. Data for tips involved in intercalary adhesions are presented in Table VI.

Chromosome substitution

The distinguishing feature of the adhesion pattern in the Oregon-R strain as contrasted with the Swedish-b lies in the considerably higher frequency of the X-2L combination of chromosome ends (20.5 per cent as compared to 2.0 per cent) and lesser frequency of the 2R-3R combination (30.5 per cent as compared to 51.0 per cent). The eight other possible combinations of two chromosome ends occur with an approximately equal frequency in both strains. What will be the reaction of the Swedish-b X (which adheres to Sw.-b 2L in only 2 per cent of the adhesions observed) with an Oregon-R 2L that is accustomed to adhering to X in 20.5 per cent of the cases? The following experiment was designed to test the effect of a Sw.-b X chromosome upon the adhesion pattern of the Ore'R chromosome by substituting a Sw.-b X for an Ore'R X, leaving the autosomes entirely Ore'R. In order to achieve the substitution of one chromosome in a strain it is necessary genetically to mark each chromosome in such a manner that it can be identified and also to prohibit crossing over so that no alteration in the chromosomes can occur. If this is achieved, the result should be a strain of Drosophila entirely homozygous for Ore'R chromosomes with the exception of the X chromosomes which are homozygous Sw.-b. The obvious objection was foreseen, that regardless of the results of such a substitution no distinction could be made between the effect of the foreign chromosome as a whole and the effect of merely the foreign tip. It was therefore necessary to compare simultaneously a stock homozygous for Ore'R chromosomes with the exception of the tip of the X chromosome which would be homozygous Sw.-b. The creation of such a stock required crossing over at the tip of the X chromosome but under such conditions that the entire complement of chromosomes would remain Ore'R with the exception of this small terminal region. As a control to compare with these two altered stocks, it was necessary to recover from the crosses a stock carrying a complete homozygous set of Ore'R chromosomes. These three stocks which the experiment is designed to create may be pictured diagramatically as follows (the small fourth chromosome is ignored in these experiments):

TABLE VI

Chromosome end	End involved in intercalary		End involved with end	
	Strain		Strain	
	Ore'R	Sw.-b	Ore'R	Sw.-b
X	54	35	28	16
2L	7	3	13	4
2R	26	41	21	34
3L	8	6	7	8
3R	4	15	29	38

	X chromosome		Chromosome	
	Tip	Remainder	2 and	3
1.	Ore'R	Ore'R	Ore'R	Ore'R
2.	Sw.-b	Ore'R	Ore'R	Ore'R
3.	Sw.-b	Sw.-b	Ore'R	Ore'R

The genetical technique used to achieve these three stocks follows: In order to maintain the autosomes from one generation to another without losing their identity, chromosomes with dominant markers and inversions in both arms were carried heterozygously with the wild-type chromosomes (Cy al^2 L^4 sp^2 and Mé, In (3R)C, Sb e l (3) e). For ease in referring to these stocks, they are abbreviated in this paper as Cy L and Mé Sb, respectively. In order to obtain the proper crossover near the tip, an X chromosome marked with recessive mutants was used (sc ec cv ct^6 v s^2 f car bb^l (abbreviated sc ec cv . . .). A crossover between scute (sc) and echinus (ec) would be in the 2 or 3 region of the salivary map or distal to 5.5 on the genetical map. This is relatively near the end of the X and would leave about 9/10th of the chromosome intact with only the distal 1/10th replaced. Other stocks used as markers were: y Hw d149 m^2 g^4 (abbreviated d149), Cl B, and Cy/Pm, ds^{39k}; H/C, Sb (abbreviated Cy/Pm; H/Sb). The series of crosses is summarized in Table VII.

TABLE VII

P_1	Sw.-b x sc ec cv . . . x Ore'R
F_1	$\frac{\text{sc ec cv . . .}}{\text{Sw.-b}}$ x sc ec cv . . . $\frac{\text{sc ec cv . . .}}{\text{Ore'R}}$ x $\frac{\text{Cy}}{\text{Pm}};\frac{\text{H}}{\text{Sb}}$
F_2	$\frac{\text{Sw.-b — ec cv . . .}}{\text{sc ec cv . . .}}$ x sc — Ore'R; Pm; H
F_3	sc ec cv . . . x $\frac{\text{Sw.-b — ec cv . . .}}{\text{sc — Ore'R}}$; Pm; H x Ore'R
F_4	Ore'R x $\frac{\text{Sw.-b — Ore'R}}{\text{sc ec cv . . .}}$; Pm; H d149; Cy L; Mé Sb x Sw.-b — Ore'R; $\frac{\text{Pm}}{\text{Ore'R}};\frac{\text{H}}{\text{Ore'R}}$
F_5	Sw.-b — Ore'R; $\frac{\text{Pm}}{\text{Ore'R}};\frac{\text{H}}{\text{Ore'R}}$ x $\frac{\text{Sw.-b — Ore'R}}{\text{d149}};\frac{\text{Cy L}}{\text{Ore'R}};\frac{\text{Mé Sb}}{\text{Ore'R}}$
F_6	$\frac{\text{Sw.-b — Ore'R}}{\text{Sw.-b — Ore'R}};\frac{\text{Ore'R}}{\text{Ore'R}};\frac{\text{Ore'R}}{\text{Ore'R}}$

In order to establish a stock which has an entire Sw.-b X chromosome with Ore'R autosomes, and a control stock with a full complement of Ore'R chromosomes, the procedure diagrammed in Table VIII was used.

TABLE VIII

P_1	Sw.-b x d149; Cy L; Mé Sb	d149; Pm; H x Ore'R
F_1	d149; $\frac{\text{Cy L}}{\text{Sw.-b}};\frac{\text{Mé Sb}}{\text{Sw.-b}}$ x Ore'R	$\frac{\text{d149}}{\text{Sw.-b}};\frac{\text{Cy L}}{\text{Sw.-b}};\frac{\text{Mé Sb}}{\text{Sw.-b}}$ x d149; $\frac{\text{Pm}}{\text{Ore'R}};\frac{\text{H}}{\text{Ore'R}}$
F_2	$\frac{\text{d149}}{\text{Ore'R}};\frac{\text{Cy L}}{\text{Ore'R}};\frac{\text{Mé Sb}}{\text{Ore'R}}$ x Ore'R; $\frac{\text{Cy L}}{\text{Ore'R}};\frac{\text{Mé Sb}}{\text{Ore'R}}$	$\frac{\text{d149}}{\text{Sw.-b}};\frac{\text{Cy L}}{\text{Ore'R}};\frac{\text{Mé Sb}}{\text{Ore'R}}$ x Sw.-b; $\frac{\text{Cy L}}{\text{Ore'R}};\frac{\text{Mé Sb}}{\text{Ore'R}}$
F_3	$\frac{\text{Ore'R}}{\text{Ore'R}};\frac{\text{Ore'R}}{\text{Ore'R}};\frac{\text{Ore'R}}{\text{Ore'R}}$	$\frac{\text{Sw.-b}}{\text{Sw.-b}};\frac{\text{Ore'R}}{\text{Ore'R}};\frac{\text{Ore'R}}{\text{Ore'R}}$

After these three stocks were obtained, salivary smear preparations were made and 100 cases of terminal adhesions recorded from each. Table IX presents the data comparing the effect on the Ore'R pattern of terminal adhesions of a Sw.-b X chromosome versus a Sw.-b X chromosome tip versus an Ore'R X chromosome.

An examination of the data shows that the Sw.-b X (which seldom adheres with a Sw.-b 2L) when placed with an Ore'R 2L (which normally adheres frequently with Ore'R X) seldom adheres with it (column 2, Table IX). This introduced chromosome brings with it into the Ore'R strain the specificities which characterized it in the Sw.-b strain. Therefore the tendency of a chromosome to adhere or not to another chromosome is a property of the chromosome itself and not of the group of chromosomes as a whole.

But the experiment also makes possible a deeper analysis of chromosome behavior. When we are dealing with a chromosome complement of Ore'R in which only the tip of the X chromosome has been derived from the Sw.-b strain, there is still little adhesion between such an X and the left arm of chromosome 2 (column 3, Table IX). The strong attraction between the X-chromosome and 2L which is so typical of the Ore'R stock thus is abrogated by the substitution of a very small terminal portion of the X from the Sw.-b strain. It is therefore in the tip region of the chromosome that its adhesion properties are localized and the body of the chromosome as a whole is not responsible for the effect.

Table IX

Chromosome combinations	1 Ore'R X with Ore'R autosomes	2 Sw.-b X with Ore'R autosomes	3 Sw.-b tip on Ore'R X with Ore'R autosomes	4 Sw.-b X with Sw.-b autosomes
X-2L	17	1	1	1
X-2R	4	1	6	14
X-3L	10	7	5	0
X-3R	20	9	0	21
2L-2R	2	5	3	2
2L-3L	3	8	10	3
2L-3R	3	2	5	1
2R-3L	5	7	8	3
2R-3R	30	36	39	50
3L-3R	6	24	23	5
Total cases	100	100	100	100

Finally it is to be noted that the X from Sw.-b (be it the whole X or only the tip replacing the Ore'R tip) fails to adhere frequently also to 3R of Ore'R. Since the X chromosomes of both stocks have a strong tendency to adhere to 3R if the latter is from the same stock, this must mean that the properties involved in the X-3R combination in one stock are different from those involved in the other stock.

Since two of the most frequent types of adhesions (X-2L; X-3R) are practically eliminated by introducing a Sw.-b X (or tip) into the Ore'R environment, it would be expected that the total frequency of adhesions should be reduced. This is not the case. The total number of adhesions per nucleus remains the same as in the parental strains (22 per cent of the nuclei contain adhesions in the Sw.-b tip stock; 20 per cent in the parental stocks). Thus the other eight possible combinations of chromosome ends (two-by-two) must rise in frequency to compensate for the two missing categories. Do they rise proportionally as might be expected? The answer is clearly that they do not rise proportionately. While some combinations occur with an unaltered frequency, others are encountered much more often, and notably 3L-3R which soars from 6 per cent to 24 per cent. The indications seem to be that 3L and 3R, since they remain structurally unaltered, had the ability to adhere in the Ore'R strain but this was never realized due to a stronger attraction between X and 3R. In other words, once the stronger attraction is eliminated by bringing in a foreign X, the weaker attraction becomes the strongest remaining. (The word "attraction" is used throughout in a purely descriptive sense and in no way is an attempt made to imply physical forces.)

Thus, by substituting a foreign X chromosome and a foreign X chromosome tip into the Ore' R strain, the locus of specificity involved in terminal adhesion is found to be the end of the chromosome. The experiment also reveals unsuspected potential attractions carried by the chromosome ends—attractions which result in adhesions only in the absence of stronger attractions.

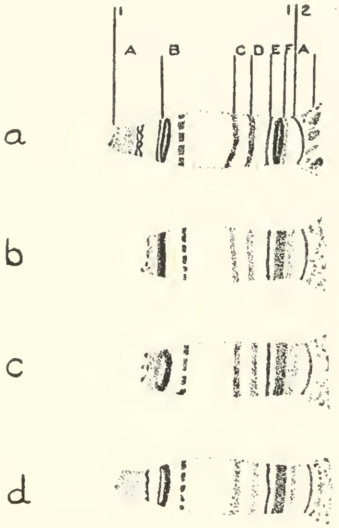

FIGURE 4. The distal end of the X chromosome in: *a*. Oregon-R. *b*. Swedish-b. *c*. Swedish-b on Oregon-R base. *d*. Marker stock.

Cytological examination of the chromosome ends

Detailed study was made of the banding at the end of the chromosome in the Sw.-b strain, the Ore'R strain, the Sw.-b tip on the Ore'R X base, and the marker stock used to obtain the crossovers (sc ec cv ct^6 v s^2 f car bb^l). This study demonstrated that the Ore'R and the marker stock X chromosomes are longer by a noticeable amount than either of the two Sw.-b X chromosomes. A study of Figure 4 shows that the Ore'R and marker X chromosomes have regions 1A 1-2-3 (Bridges map) while the others lack this. Whether this is a typical terminal deficiency as described in connection with the micromanipulation data in a previous section of the present paper, or not, the fact remains that there is a definite morphological difference in the X chromosome tips in these strains and furnishes evidence in addition to the genetical evidence that the tip of the experimental stock is truly Sw.-b. It becomes obvious to suggest that the different specificities in terminal adhesion shown by the Ore'R and Sw.-b chromosomes may be due to the morphological difference. It was previously suggested (Hinton and Atwood, 1941) after a study of adhesions in a terminally deficient stock, that there are subterminal layers of properties which can operate if the tip is removed and the raw end heals. The new end has the ability to assume the role of the previous end, but not with the identical specificities.

Discussion

Since all cells of an individual have the same descent one need not look to a difference between the chromosomes in order to explain the differences in adhesions among the cells of a single gland. Yet we also know from the experiments in the present paper that the end of the chromosome is the important factor in deciding the types of adhesions. This has meaning if we conceive of the tip as containing several properties for sticking, one of which, for example, involves a strong attraction for some other chromosome while another properly involves a weak attraction for still a different chromosome. If, at some stage in the development of the salivary gland, there is a movement of the chromosomes and an approximately equal chance that any chromosome end might pass close to any other chromosome end in the nucleus, it is logical that the two ends which have a stronger attraction for each other will show adhesion more often than will two ends with a weak mutual attraction which necessitates a closer approach before sticking can occur. This is suggesting that the different properties at the end of the chromosome responsible for the sticking have different sensitive volumes, or spheres of influence in which they are capable of attracting.

The tip replacement experiment furnishes the best clues as to the number of properties which must be assumed to be at the end of the chromosome. It is obvious from the data that all tips do not contain the same properties even though any two have some in common. Also, one end may exert an influence over another end so strong that weaker attractions are seldom realized except in the absence of the stronger attraction. Therefore the chromosome end must be assumed to contain properties seldom expressed due to the presence of more dominant properties. With these indications in mind it is possible to construct a model of the ends and the properties they contain. If the ends in Ore'R are designated as X-CA, 2L-CD, 2R-Bad, 3L-ac, 3-R-ABc; and if it be assumed that the letters used represent properties of the chromosome ends, and that the attraction between A and A is greater than between A and a which in turn is greater than between a and a, etc.; then the model expresses the various frequencies shown by the data.

Such a model is mainly a diagrammatic restatement of the data, but its main value is to indicate the amount of assumption necessary for a working hypothesis. According to this most simple model it is necessary to assume three or four properties per tip in order to include all data, and at least six different properties. One of these properties may be chromosome length, and another a general attraction between all parts of the chromosomes, but the rest of the properties must be assumed to be more specific in nature. These properties can be assumed to be either

TABLE X

(Assuming CC produced 20 per cent adhesion; Cc, 1 per cent; cc, 1; AA, 20; Aa, 6; aa, 1; BB, 20; dd, 3.)

Combination	Attraction due to:	Frequency	
		Expected	Actual
X-2L	CC	20	20
X-2R	Aa	6	8
X-3L	CC, Aa	7	6
X-3R	AA, Cc	21	22
2L-2R	dd	3	3
2L-3L	Cc	1	1
2L-3R	Cc	1	1
2R-3L	aa	1	1
2R-3R	BB, Aa	26	30
3L-3R	Aa, cc	7	6

characteristics of a single substance or units of material at the end of the chromosome.

The idea that the whole phenomenon of terminal adhesions is due to a single heterochromatic attraction becomes impossible in light of the data presented in this paper. If quantitative differences in a single factor were responsible for the different frequencies of adhesions, as is assumed by the heterochromatic theory, then a mathematical model could be set up to fit. Such a model is impossible to formulate for one cannot explain in simple quantitative terms (with the adhesion frequencies following either the sum or the product of varying quantities) such frequencies of combinations of chromosome ends as have been shown to exist by the data. If heterochromatin is the substance involved in the formation of terminal adhesions, it becomes necessary to assume at least six types of heterochromatin either with stronger attractions between some types than between others or any one type possessing several properties. The same objection holds for the telomere theory. The telomere would have to consist of many parts each with different specificities. But regardless of whether we conceive of the end of the chromosome as being heterochromatins, compound telomere, or genic material, the indications are that it shows several specific attractions of various strengths, dependent upon proximity for expression, subject to change by mutation, and able to reform if removed or altered.

SUMMARY

In order to obtain further insight into the nature of chromosome ends, the phenomenon of terminal adhesions in salivary gland chromosomes was studied.

Salivary gland chromosomes involved in terminal adhesions were stretched with the needles of a micromanipulator until the ends broke apart. No case was found in which the ends had gained or lost a band.

From determining which chromosomes were involved most frequently in adhesions, it is concluded that the frequency with which any two chromosome ends adhere together is non-random, differs in different strains, is subject to change

over a period of time, is not affected by the genotype of the cytoplasm nor temperature. However, the total incidence of adhesion is affected by temperature.

Intercalary regions associated with chromosome ends were found to be nonrandom.

A foreign chromosome end introduced into a strain demonstrates that the specificities shown in terminal adhesions are characteristics of the end itself, and reveals potential abilities of ends which are not realized under normal conditions.

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THE ARMY-ANT BEHAVIOR PATTERN: NOMAD-STATARY RELATIONS IN THE SWARMERS AND THE PROBLEM OF MIGRATION

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The problem of migration occupies a position of considerable importance in the study of animal psychology, involving as it does questions such as "instinct," reproductive functions influencing behavior, sensory and ecological factors in behavior, and others of significance. Numerous writers have suggested, with reason, that the scientific explanation of animal migration may be improved not only through further investigations of the best known migrants, among birds and fishes, but also by studying other instances wherever they arise in the phyletic series. By investigating migration and related patterns in a variety of animal types, it may well be possible to clarify both the essential fundamental characteristics of migration and characteristics which may differ or be absent according to the given biological setting. Accordingly, our present discussion introduces such an exercise in the study of one of the social insects.

The most characteristically pedestrian of all insects, species of the ant subfamily Dorylinae in the American tropics, commonly known as "army ants," seem committed to a wandering of their colonies without any possibility of more than relatively brief nesting stops. In this respect they resemble their Old World relatives, the "driver ants," of which Savage (1847) said:

> "From its locomotive habits the impression . . . has obtained, that it has no fixed habitation. This my observations go to confirm in respect both to their appearing and disappearing from certain localities, and the absence of cells or magazines."

The typical inability of doryline colonies to settle down permanently has prompted many writers (in particular: Sumichrast, 1868; Belt, 1874; Vosseler, 1905; and Wheeler, 1910) to refer to these ants as "migratory." It is in order to inquire whether the application of this term to doryline colony movements is merely gratuitous, as other writers such as Heape (1931) maintain, or is actually justified in a technical sense.

Systematic observations soon reveal that the rambling of doryline colonies is not random or haphazard but instead involves a predictable routine describable as a regular pattern. In a previous paper (Schneirla, 1938) such a pattern has been described for *Eciton hamatum,* the type species of the American subgenus *Eciton s.str.* In the season of rains, evidently the time of optimal Eciton activity, this pattern exhibits two prominent activity phases: one, a nomadic period, in which there are large daily raids, each passing at nightfall into a movement of the entire colony to a new temporary nesting site, and two, a statary period, in which small daily raids are staged from a stable bivouac site, without any bivouac-change movements. These major changes in colony behavior occur in regular succession during the rainy season, under conditions indicating that the key factor in the behavior cycle of a colony must be present or absent in dependence upon the condition of developing broods. The pace-setting basis of the entire system of events may be traced to the reproductive properties of the *E. hamatum* queen (Schneirla, 1944b).

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To what extent does this pattern of events prevail in other dorylines, particularly in species which deviate noticeably from the type species in prominent aspects of behavior? *E. hamatum* is prominent in the forests of tropical America because it is a terrestrial species staging raids which involve far-flung systems of ramifying columns headed by small groups of predators, i.e., it may be called a column raider. Strikingly different are the swarm raiders, whose forays are headed by immense unitary bodies of ants rather than by innumerable small spearhead groups. It is typical in *E. burchelli,* which we have studied as a representative of the swarm raiders, that only one principal trail connects the primary raiding masses with the colony bivouac. Even so, burchelli colonies undergo a bivouac-change movement as sequel to a raid much as do hamatum colonies. There are radical differences between these species in the actual mechanism of the exodus as an outcome of raiding, yet we have found (Schneirla, 1944a) that certain fundamental events are common to both.

From this arises the principal subject of the present paper, the larger picture of relationships in the life and behavior of swarm-raiding burchelli colonies over considerable periods of time. If burchelli colonies undergo a cycle of movement and pause somewhat like that of hamatum, it is desirable to find how the phases of this cycle occur, and what their underlying causes may be. In that sense, the present investigation represents a test of the theory already advanced for the Eciton type species.

An interest in the causation of Eciton mass movements inevitably leads to questions concerning their relationship to the general occurrence of collective movement in animals. In the general literature this relationship is not at all clear, in particular because writers on animal movements have based their conclusions concerning the dorylines on doubtful and incomplete evidence.

In one of the most comprehensive treatises on the subject of animal movements, Heape (1931) distinguishes between two types of "voluntary" (i.e., active) movements: one, "elemental" or "climatic" movements, "undertaken in search of food or water, or in order to escape from unfavorable climatic conditions," and two, "gametic" movements, which are "impelled by impulses which arise from physiological activity in the reproductive system, and concern the life, that is to say, the continuity of the race" (p. 336). Heape insists that gametic (i.e., reproductive) causes are essential to true migration, ". . . a movement which involves a journey to a definite area, and a return journey to the area from whence the movement arose." Distinguished from this is emigration, which rests upon alimentary or other non-gametic causes, and in which a return does not occur.

On this basis, Heape believed that doryline movements should be classed as non-migratory, as cases of emigration. For example, Eciton colonies, which "constantly change their home and hunting ground," are not accepted by him as valid instances of nomadism, since

> ". . . these animals recognize no territory; they travel wherever it may suit them to go, establish themselves with their pupae wherever it may suit them to stay, and remain there as long as sufficient plunder is to be found within the limit of their raiding capacities. Moreover, they do not return to any permanently established home" (p. 178). "I judge them to be emigrants, whose sole business in life is raiding. They have a temporary home only, it is true, but still a home within a definite territory so long as it lasts; that is, so long as their ravages over that territory bring them in what they need. Then they move on somewhere, anywhere else, with their young; make there another home, and again ravage the territory available around."

This view of doryline movements, although misrepresentative (Schneirla, 1944a), appeared to find corroboration in the literature. From Carpenter's (1920) observations of day-long movements of Dorylus colonies in East Africa, "streaming along . . . to a new hunting ground, where they quickly form a home and establish themselves," Heape draws a distinction between such activities and "minor streams of hunting parties, which are formed as occasion demands after the colony has settled down in new territory." But, he says,

> "while emigrating these ants do not carry on raids, they are concerned only in traveling to a new home. Presumably they have sufficiently divested the region around their last home, they have worked out that region, and now seek new territory to ravage, and being raiders, and living only by raiding, they disregard all territorial rights of others and establish themselves, where spoil promises to be plentiful."

This has been the prevalent view, that doryline colonies move from place to place rather haphazardly, purely on an alimentary basis. From his observations of Eciton behavior in Nicaragua, Belt (1874) stated that ". . . it is a curious analogy that, like the hunting races of mankind, they have to change their hunting grounds when one is exhausted, and move on to another" (p. 17). Vosseler (1905), having studied the doryline species *Anomma molestum* of East Africa, stated that a colony occupies the same nest until it has destroyed all of the available prey in a locality, which requires some 8 to 10 days, then the colony moves elsewhere. Once the vicinity of the nest has been thoroughly ransacked, when the haul of booty no longer suffices for the food needs of the colony, "so muss es sich zur Auswanderung entschliessen" (p. 293). Vosseler's account has led (or, we might say, "misled") numerous secondary writers, including Forel (1923), to adopt the food-exhaustion explanation of doryline movements. Reliance upon the presumed soundness of this interpretation brought Heape (1931) and Fraenkel (1932) in particular to the decision that Eciton movements are not to be considered instances of migration.

The last two writers, especially, might have been less positive concerning the status of Eciton movements had they not overlooked an important study by Müller (1886). Because this study is the only one preceding our own which contains information about the day to day activities of an Eciton colony (an *E. burchelli* colony, moreover), and because it is published in a relatively inaccessible journal, it is desirable to offer here a detailed summary of the pertinent results.

Müller opened his record on Feb. 28, 1885, when a burchelli colony came to notice in his brother's garden near Blumenau, Prov. St. Catherina, Brazil, and ended about three weeks later. A summary of his observations follows:

Feb. 28 (on the two preceding days the ants had been seen raiding in the garden) at 9 A.M., when discovered on Feb. 28, the ants formed a closed column (very probably what we have termed the "principal column"—1940). Having viewed "all of the branches," Müller found headquarters beneath the branches of a fallen tree. Some of the larvae were enclosed in cocoons, but none had pupated. At 8:00 P.M. when the spot was revisited, a strong column of Ecitons was removing larvae with all signs of dispatch, moving in the reverse direction to that of the morning (i.e., away from the bivouac). At 11:00 P.M. the procession was still in full progress.

Mar. 1—The column was traced at 7:00 A.M. to a fallen hollow tree, into which the ants disappeared.[2] Since there were no traces of other columns from this site, which was about 200 paces from that of Feb. 28, Müller concluded that the hollow tree must contain the new nest.

Mar. 2.—At 6:00 A.M. the ants were engaged in changing quarters once more, this time having taken a fallen hollow tree-trunk 50 paces from the last one.[3] On this day also the ants went out pillaging, raiding to the east.

Mar. 3.—At 6:00 A.M. no ants were to be seen; a few hours later they were occupied with booty, on a trail to the southeast.

Mar. 5, 6—At 6:00 A.M. no ants were seen outside the nesting place.

Mar. 7—At 10:00 A.M. there was a single column, raiding almost directly westward. "Thus, in visiting the surrounding area on different days, the ants appeared to proceed fairly systematically."

Mar. 8—Despite good weather, the ants were not seen outside the tree when the site was visited at 7:30 and 11:00 A.M., and at 4:00 P.M.

Mar. 9—When no signs of activity were observed, smoke was introduced, forcing the ants to move into the lower portion of the trunk. No sexual forms were seen. All visible larvae appeared to be enclosed.

Mar. 10—The former place was deserted; finally the colony was found hanging in a cluster against the inner wall of a standing tree close by. (There is no mention of a raid.)

Mar. 11—No booty column was observed.

Mar. 12—When the site was visited toward evening, a meager raiding column was seen.

Mar. 13—The tree trunk was opened, and large numbers of cocoons were seen, but no fertile forms.

Mar. 14—The colony was anaesthetized and a considerable part of it, estimated at about 5,600 cu.cm. in volume, was taken away in a sack for examination. Less than half of the workers and about half of the brood were removed. Among hundreds of individuals examined, the brood contained only enclosed pupae, with the exception of a still unpupated small worker.

Mar. 15—In a further exploration of the cluster in the hollow tree, masses of eggs were discovered.

Mar. 16—In the afternoon a weak column was observed, out for booty.

Mar. 17—The hollow tree was knocked over and the ants once more were driven out with smoke. A search failed to disclose the queen. This marked the end of the study.

In discussing his results, Müller focussed upon his observation of a decline in raiding after March 8, the day when (as he judged) the last larvae were enclosed.

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[2] On the morning of March 1 there was observed at one side of the burchelli column a wingless male, half led and half pushed along by a few workers. This individual was accepted by a group of workers with whom he was placed, and hence must have belonged to the colony. Subsequent examination by Forel disclosed that this burchelli male actually was a Labidus, one of the group whose biological affiliations had been long in doubt.

[3] It is important to note that this was the last active bivouac-change movement of Müller's colony during the period of observation.

This parallel between decreased raiding and a change in brood condition seemed to him far from accidental, since "a colony needs less food once its brood is enclosed, the ants raid correspondingly less" at such times. Although this explanation of reduced raiding based upon an inferred reduction of food requirements appeared to be justified by the observed circumstances, two important points relevant to its validity were not considered in the discussion. These were, first, that important changes in brood condition must have occurred well before March 9, and second, that a considerable decrease in raiding must have taken place about March 2. Such facts lead us to a consideration of events beyond raiding, favoring a different explanation than the one Müller offered.

Other events in colony behavior such as the matter of changing the nest-site appear to have received a very separate consideration in Müller's thinking. To him "the helplessness, the difficulty, with which they settled upon a change of dwelling after March 9," in contrast to earlier changes of quarters, could be explained only by assuming that a fertile queen was present (i.e., after March 9). Heavy and difficult to transport, he thought, this individual must have been a hindrance to removals. Although she must have been present on Feb. 28, the queen then very probably was "slighter and more easily transported." A direct contradiction of this line of reasoning exists in Müller's own evidence, in the fact that the colony kept to the spot even after the queen apparently had laid her eggs and might therefore have been "easily transported." To add to the difficulty, his own record shows that the colony first settled in place not on March 9, but actually on March 2. The facts call for a very different type of explanation.

Thus Müller advanced two distinctly different hypotheses, one to account for changes in raiding behavior, the other to explain colony immobility. The first of these is significant, not because it is valid as an explanation, but because it suggests that a relationship may exist between colony behavior and observable conditions within the colony. It was with lively interest that the present writer first read Müller's paper after having prepared an account of his own initial Eciton investigation (1933) in which the existence of such a relationship was noted and its significance indicated.

A final statement deserves to be quoted:

> "Wenn nun das Vorhandensein einer Königin ein Hindernis für das Weiterwandern, die Zeit der Puppenruhe die Zeit eines viel geringeren Nahrungsbedürfnis ist, bei dem das sonst in wenigen Tagen abgesuchte Terrain wohl Wochen lang genügt, dann erscheint dies zeitliche Zusammenfallen zweier nicht in direkten Zusammenhang stehender Ereignisse (Verpuppung und Beginn der Eiablage) nicht als etwas Zufälliges, sondern als durchaus notwendig."

This suggests that Müller glimpsed the unity of the entire phenomenon, although the fixity of his two disparate hypotheses prevented him from reaching a common basis of theoretical explanation.

One relationship in the complex Eciton pattern about which a clear understanding is essential concerns the bearing of raiding activities upon colony movement. Of course, in the form of the behavior and in the time of day at which these two activities occur there are important typical differences, as a number of writers (Bates, 1863; Müller, 1886; Carpenter, 1920) have pointed out for various dorylines. From his observations of the African species *Dorylus nigricans* and *glabratus* Carpenter (1920) distinguished raiding forays from other movements. "But every column of Ensanafu that one sees is not engaged in changing camp; most often they are either going out to hunt, or returning laden with spoils." We have shown in detail for burchelli (1944a) that raiding is organized in the morning and develops during the day, whereas the bivouac-change movement occurs at the end of day and during the night. Yet to differentiate raiding from colony movement should not lead to overlooking the relationship of these events, as Müller did in offering distinctly different explanations for them.

As a foundation for the present paper, our previous consideration (1944a) of the manner in which raiding leads into colony movement under given conditions is materially important. For in that connection it was shown that under certain other conditions a raid *cannot* eventuate in a change of nest site. The solution lies in the fact that very different conditions exist within the colony at the two times, as we have demonstrated in the case of the column-raiding *E. hamatum* (1938; 1944b). Although the form of burchelli raiding differs materially from that of hamatum forays, the fact that in both species colony movement occurs as the outcome of raiding suggests the existence of further similarities.

The purpose here is not merely to compare the nomadic activities of two Eciton species, although this is a useful way to extend our explanation of the Eciton problem as such. Beyond that, we are led to an interest in how Eciton movements fit into the general picture of the mass movements of animals. It will be useful to consider whether the Ecitons are truly migrant, in the sense that they may possess the most essential features of animal migration in general, or whether certain criteria generally considered essential to migration proper are lacking in these insects.

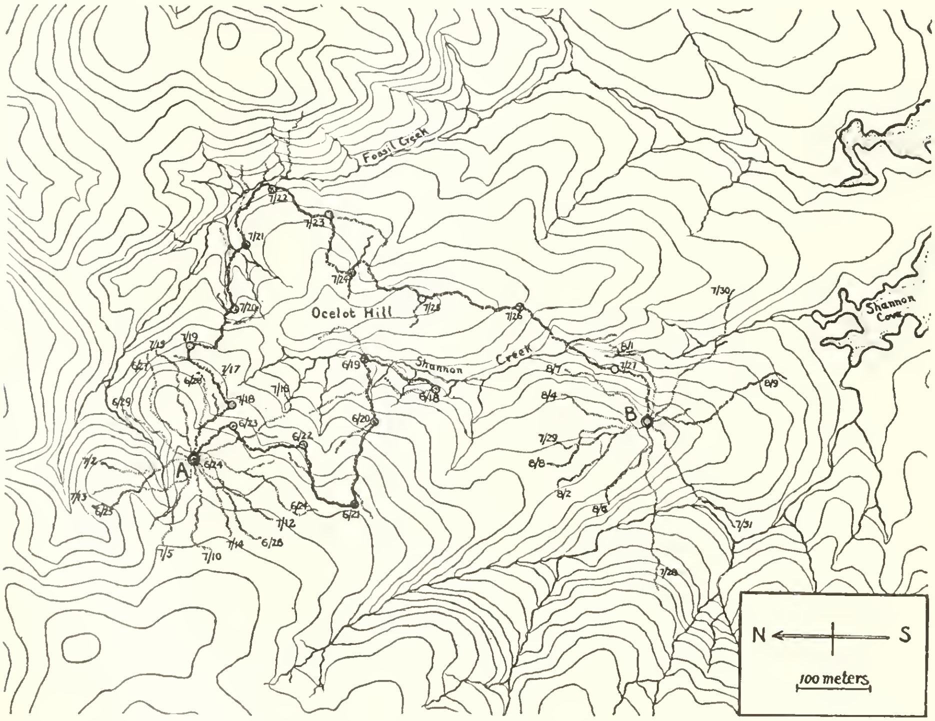
This study was conducted at the Barro Colorado Island biological reservation in Gatun Lake, Panama Canal Zone. The records were obtained during the rainy months May to September, for the most part during the years 1936 and 1938. The method was largely one of field observation, supplemented by special observations and tests in the laboratory. The general plan was to keep certain colonies on record over a considerable period of time, in order to study changes in their external behavior and internal condition during the interval; and correlatively, to obtain records of similar events in other colonies over relatively short periods of two or more days.

Results

Activities of a representative colony of E. burchelli: This colony of *E. burchelli* (record number 38I) was first encountered on the morning of June 18, 1938, when it was engaged in a vigorous swarm raid. That evening it moved to a new bivouac site, and subsequently the study of this colony became the chief problem of the daily routine. The period of study ended on August 10 with the writer's departure from the Island, a total of 53 days in which colony 38I was under daily observation. During that time the colony moved over rugged terrain through forest a distance of nearly two miles, in an irregular path.

The manner in which the colony operated during the period of study is sketched in Figure 1, in which are represented the various bivouac sites successively occupied, the principal lines of most of the daily raids, and the routes of successive bivouac-change movements. The principal facts concerning the activities of colony 38I are given in Table I. The following discussion of this record is divided into sections according to certain major changes which occurred successively in the behavior and condition of the colony.

Period 1 (nomadic): When the study opened, for a few days the colony staged very large daily raids, each passing over toward evening into an exodus which ended sometime during the night after the entire colony had moved to a new bivouac site. Activities began promptly each morning at daylight with the divergent spreading of ants around the bivouac, and matters progressed so that a directionalized swarm was formed within less than two hours. The swarms that developed commonly exceeded 10 meters in width by 11:00 A.M., and became somewhat unwieldy and disorganized about that time, so that one or more divisions subsequently occurred resulting in two or more sub-swarms moving divergently. The outcome was a complex raiding system to be observed in the afternoon, with sub-swarms operating more or less independently although interconnected in the rear by various secondary columns, the latter discharging into a principal column communicating with the bivouac. The forays developing on the first six days of this study, up to and including June 23, conformed essentially to the pattern of "maximal" burchelli raiding previously described.[4]


Fossil Creek
7/22
7/23
7/21
7/24
Ocelot Hill
7/25
7/26
7/20
7/15
7/19
6/27
7/17
6/28
7/16
7/18
6/29
6/19
Shannon Creek
6/18
6/20
6/23
6/22
7/2
A
6/24
7/13
6/25
6/24
6/21
7/12
7/14
6/26
7/5
7/10
7/30
Shannon Cove
8/1
7/27
8/7
8/4
8/9
B
7/29
8/8
8/2
8/6
7/31
7/28
N
S
100 meters

All six of the bivouac clusters formed successively in this brief period were located in the open and close to the ground. Typically the colony formed a cylinder which hung down about 40 cm. to the ground from where ants in the upper fringes of the wall were securely hooked by their tarsal claws to the lower surface of a log or mass of vines. Two of the bivouacs were pouches formed against trees one or two meters from the ground.

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[4] The descriptive statements of this section concerning the development of a raid in *E. burchelli* are consistent with the results of a detailed analytical study of swarm raiding previously reported (Schneirla, 1940).

Generally in the middle of the afternoon or if delayed, by dusk, a large-scale exodus was recognizable, as a rule monopolizing the route of the principal raiding trail. In three of the six instances this exodus during the afternoon was blocked from the principal trail by return traffic, and developed a new route eventually followed by the colony movement. The rise of a bivouac-change movement as an outcome of burchelli raiding has been described in some detail (Schneirla, 1940, 1944a).

The transfer generally was completed before midnight, except as on June 23 when afternoon traffic difficulties delayed the beginning of the heavy exodus until early evening.

The terminal removal of the series took the colony to a hollow tree only about 45 meters from its previous bivouac site. Within this tree it clustered at a considerable distance from the ground. From other observations it can be said that this last movement of a nomadic series generally is distinguished from preceding ones, as in this instance, by the relative shortness of the trek as well as by the nature of the bivouac site. Under these conditions the colony tends to cluster well above the ground, commonly within a hollow tree or similar secluded situation.

The queen was not seen during this series of moves. In each of four instances when the queen of another burchelli colony has been observed under conditions comparable to these, this individual was well contracted and (appearing in the last third of the movement) was able to force a way despite the crowding of excited workers around her.

Period 2 (statary): On the night of June 24 the colony formed an elongated pouchlike cluster high (ca. 10 meters up) in the interior of a large lightning-cleft tree. The bivouac remained in almost exactly the original position (where it was well sheltered from the elements) until July 16, i.e., for a period of slightly more than three weeks.

During this interval of immobility raids when they occurred were definitely smaller than before June 24, fitting the picture of "minimal" raiding previously described for burchelli (1944a). The forays were always slow to begin in the morning (although before July 24 activities began at dawn) and occasionally did not begin until afternoon. On some of the days, as Müller observed in his colony, there was no raiding at all. This interval of frequent raidless days began about one week after the time of the last movement, roughly in the middle of the period. When raids occurred they were relatively small, without major swarm divisions such as those commonly observed in nomadic burchelli colonies.

In the early afternoon of each raiding day there occurred a recognizable resurgence of ants from the bivouac into the raiding system, although this afternoon exodus was definitely weaker than in raids preceding June 24. Also, a more or less extensive return to the bivouac typically was under way at dusk. After July 13, when the raids increased noticeably in strength, the return to the bivouac frequently was observable until after midnight.

TABLE I

Changes in the activity and condition of *E. burchelli* colony 38I between June 18 and August 10, 1938 (cf. Figure 1)

Date	Bivouac	Condition of the brood	Raiding activity	Behavior toward end of day
June 18	A cylinder hanging from brush to ground.	Single brood: moderately developed larvae.	A well-developed foray to the *N*, with swarm division. New push from bivouac in afternoon.	Somewhat delayed bivouac-change, over new route to *N*, *NE* started in afternoon.
June 19	A flat low cylinder beneath a log.	Single brood: moderately developed larvae.	A very active raid to *NE* × *N*; three swarm divisions.	Similar to above; movement to *S*, *SW* over route started in afternoon.
June 20	Long mass pouched against tree, one meter from ground.	Single brood: moderately developed larvae.	A vigorous raid to *NW*; two swarm divisions.	Movement to *W*, *NW* over raiding trail established in morning.
June 21	A long, bulbous mass suspended in vines, one meter from ground.	Single brood: moderately developed larvae.	Very large raid to *N*, *NE*, with swarm divisions.	Movement delayed until evening, occurred over raiding route to *N* and *NE*.
June 22	A long cluster from brush to ground.	Single brood: moderately developed larvae.	Very active raid to *N*; swarm divided three times.	Moved to *N* in late afternoon over raiding route.
June 23	Bulb-shaped mass suspended between buttressed tree roots.	Larvae close to maturity. Spinning observed.	Maximal raid to *NW*; swarm divided first at 9:30 A.M.	Short distance to *NW* over raiding route; delayed until evening.
June 24	*Site A:* A large pouch against inside wall of hollow tree, about 8 meters up.	Larval cocoon-spinning in full swing during day.	Moderately active raid to *S*, *SW*; definitely less active than on previous days.	*No movement.* All raiders had returned to bivouac before 10:00 P.M.
June 25	Site A: no change.	Cocoon-spinning observed, but less widespread.	Moderate raid to *S*, *SW*; no swarm division.	No movement; return traffic dominated principal trail after 2:00 P.M.
June 26	Site A.	Spinning less noticeable.	Moderate raid to *S*, *SW*; no swarm division.	No movement; raiders still returning at 8:30 P.M.
June 27	Site A: cluster somewhat smaller.	Spinning of outer envelopes evidently completed.	Less active raid to *NE*; no swarm division.	No movement; nearly all raiders returned before 8:00 P.M.
June 28	Site A.	Enclosed brood, still spinning inside envelopes.	Raid to *E*; small swarm, no division.	No movement; general movement toward bivouac at 5:00 P.M.
June 29	Site		Minimal raid to *N*, *NE*.	No movement; general return at 6:00 P.M.
June 30	Site A.	Brood pupating.	Not raiding at 10:30 A.M., possibly no raiding at all.	No movement. Not visited in afternoon or evening.
July 1	Site A.		Not visited.	
July 2	Site A.	Brood pupating.	Small raid to *NE*, *N*.	Return to bivouac complete by 9:45 P.M.
July 3	Site A.		Not visited.	
July 4	Site A.	Brood all pupated. New batch of eggs present.	No raiding.	
July 5	Site A.	Brood all pupated. New batch of eggs present.	Moderate raid to *NW*, *W*.	General return in progress at 2:00 P.M.
July 6	Site A.		Not visited.	
July 7	Site A.	Brood all pupated. New batch of eggs present.	No raiding.	
July 8	Site A.	Brood all pupated. New batch of eggs present.	No raiding at 10:00 A.M.	
July 9	Site A.	Brood all pupated. New batch of eggs present.	No raiding.	
July 10	Site A.	Enclosed broods; hatched eggs seen.	Moderate raid to *W*.	General return in progress at 4:30 P.M.
July 11	Site A.	A small number of cocoons opened.	No raiding.	
July 12	Site A.	A few more cocoons opened.	Moderate raid to *S*, *SW*; no swarm division.	Raiding ceased before dusk; general return during early evening.
July 13	Site A.	Further cocoon-opening.	Moderate raid to *NW*; no swarm division.	General return completed before 10:00 P.M.
July 14	Site A.	Cocoon-opening more active; continues at night.	Moderate raid toward *SW*; no swarm division.	General return completed about 11:00 P.M.
July 15	Site A: cluster appears larger.	Cocoon-opening apparently increasing.	Moderate raid toward *NE*, *E*.	General return observed at 7:00 P.M.
July 16	Site A: cluster much larger.	Cocoon-opening at its peak.	Raiding to *S*, *SE*, noticeably more vigorous.	General return still under way at 11:00 P.M.

TABLE I—*Continued*

Changes in the activity and condition of *E. burchelli* colony 38I between June 18 and August 10, 1938 (cf. Figure 1)

Date	Bivouac	Condition of the brood	Raiding activity	Behavior toward end of day
July 17	Site A: cluster still large.	Cocoon-opening still in active progress; callows on trail near bivouac.	Vigorous raid to *SE, E*, with swarm division.	Bivouac-change movement to *SE, E* over raiding trail, well under way at 4:00 P.M.
July 18	*New bivouac*, a cylinder hanging to ground from root.	Last cocoons opened, callows seen on raiding trails; brood of very small larvae seen.	Maximal raid to *NE*, with two divisions of swarm.	Movement to *NE* under way at 3:40 P.M., over raiding route.
July 19	A cylinder beneath raised tree roots.	Callows seen farther out on raiding trails.	Maximal raid to *S, SE;* swarm division at 10:30 A.M.	Movement to *SE* over raiding trail, under way at 6:00 P.M.
July 20	Cylinder hanging from vines to ground.	Callows now common on raiding trails.	Maximal raid to *E;* swarm divides twice.	Movement to *E* over raiding trail, under way at 4:45 P.M.
July 21	Two cylinders hanging from log to ground.	Larvae now observed in act of feeding.	Maximal raid to *E;* one major; one minor division.	Movement to *SE, E* under way at 3:30 P.M.; greatly delayed by marginal "traffic jam."
July 22	Cylinder hanging from raised roots to ground.	Callows seen near base of swarm.	Maximal raid to *SE, E;* swarm divides early; new push from bivouac in afternoon.	Movement to *SW, S* over raiding route of new afternoon push.
July 23	Bulb formed around small tree 2 meters from ground.	Larvae now approach full growth; callows numerous in swarm.	Maximal raid to *SW, S;* swarm divides early, at 9:00 A.M., twice later. New push in afternoon.	Movement delayed by traffic jam near bivouac, under way at 7:00 P.M., over new trail of afternoon to *W, SW*.
July 24	A cylinder between roots, resting on ground.	Large larvae now well distributed through bivouac.	Maximal raid to *SE, E* and *S;* first swarm division at 8:30 A.M.; new push to *W* begins about 3:00 P.M.	Movement delayed by first exodus to *W;* finally under way to *SE* × *S* at 8:00 P.M.
July 25	Clustered inside open end of hollow log.	Larvae nearing full maturity; a few instances of spinning.	Vigorous raid to *S;* two or more swarm divisions.	Movement delayed by return traffic; under way to *S* at 6:30 P.M.
July 26	Bulb formed against tree one meter from ground.	Larvae widely distributed through cluster. More spinning.	Maximal raid to *S, SW;* major swarm division at 9:00 A.M., two others later.	Movement delayed by traffic jam at outer junction; under way to *S, SW* at 6:00 P.M. Bivouac forming at 7:00 P.M.
July 27	Long cluster one meter from ground, around tilted tree.	Larvae widely engaged in spinning.	Maximal raid to *S*, then turns westward.	Movement over raiding trail to *S*, then *W;* delayed by traffic difficulties.
July 28	*Site B:* in upper interior of hollow tree.	Cocoon-spinning visible on shelf in tree interior.	Moderate raid on continuation of 7/27 route to *W*.	No movement; general return after raiding ceased at dusk.
July 29	Site B.	Boood subsequently inaccessible.	Less active raid to *NW, W*.	No movement.
July 30	Site B.		Moderate raid to *SE*.	No movement.
July 31	Site B.		Moderate raid to *SE*.	No movement.
Aug. 1	Site B.		Moderate raid to *E*, then bends to *N;* no swarm division.	
Aug. 2	Site B.		Less active raid to *NW*.	No movement.
Aug. 3	Site B.		No signs of raiding at 4:00 P.M.; probably no raiding at all.	No movement.
Aug. 4	Site B.		Minimal raid to *NE* × *N*.	No movement.
Aug. 5	Site B.		No raiding.	No movement.
Aug. 6	Site B.		No raiding until 2:00 P.M., then raid started to *NW*.	No movement.
Aug. 7	Site B.		Moderate raid to *N, NE*.	No movement; general return in progress at 6:00 P.M.
Aug. 8	Site B.		Moderate raid to *N*, then *NW*.	No movement.
Aug. 9	Site B.		No sign of raiding at 2:30 P.M.; raid started after 3:00 P.M. to *S, SE*.	
Aug. 10	Site B: probably occupied for another week. Observations discontinued.		Minimal raiding to *SE*, stopped by protracted rain at 3:00 P.M.	General return slowly under way after 6:00 P.M.

The colony remained at site *A* in the hollow tree from June 24 to July 17, an interval of 23 days. During this interval 15 daytime forays were observed. Each of these raids, as may be seen in Figure 1, kept roughly to a given general direction, a characteristic of burchelli raids previously discussed (1940).

When we measure the angle of divergence between the main line of each raid and that of the following raid (excepting the three instances when the colony was not visited), the values with one exception are found to lie between 30° and 160°.[5] Thus, although there was no particular order in the directions of successive raids, evidently there were factors opposing two raids in close succession into the same terrain. It is apparent from the figure that the raids were scattered fairly well in all directions around the bivouac. Such is not always the case, especially when vegetation and terrain present differences around the bivouac which may affect distribution of prey or differently canalize the first spreading of the ants in the morning. Not all instances support, as well as did this one, Müller's impression that the environment is probed in a thorough manner.

However, it must not be thought that even during a stop of 23 days at site *A* the raids of this colony even came close to depleting the supply of prey in the surrounding area. The record shows that large sectors, and even certain zones fairly close to the bivouac, were covered incompletely or not visited at all. Moreover, areas cleared of prey may be speedily repopulated. Three days after colony 38I had moved from this site, another burchelli colony staged a vigorous raid fairly close to the bivouac tree on the west, with evident signs of a large haul of booty.

Period 3 (nomadic): On July 18 there occurred a foray which clearly surpassed in vigor and numbers of participants any raid since June 24, and which ended in a bivouac-change movement along the principal raiding trail. The exodus led to a marginal site established in the evening, more than 100 meters from the long-tenanted one in the hollow tree. Thereafter the bivouac site was changed daily, as described for period one above. All clusters were formed in exposed places.

A difference worth mentioning is that whereas the bases of all of the first five bivouacs touched the ground, three of the last five clusters were in somewhat elevated positions. On July 17 in the evening the ants began a cylinder on the ground between tree roots, only to desert this for an elevated position early in the night. All raids of this period were vigorous or "maximal" ones, in which activities began promptly at dawn and one or more swarm divisions were the rule. Since the ants seldom worked back through the approach-area of the preceding day for more than two or three hours in the early morning, the major new raiding push always developed into unworked terrain in some other direction.

This interval of maximal raiding and daily bivouac-change ended on July 27, after eleven days on the march.

Period 4 (statary): A vigorous raid on July 27 terminated in a delayed night movement, relatively short in comparison with preceding movements, which took the colony to a clustering place high (evidently 15 meters or more aloft) in the upper interior of a huge lightning-cleft tree. Unfortunately the cluster lay out of sight in the dark upper interior of the tree, where it could not be illuminated or reached in any feasible way.

During the last three days of the study measures of desperation such as dust shot were used in attempts to puncture the bivouac and obtain samples of the brood, without success. When raids occurred, the ants reached the ground as a rule in one or more broad columns on the outside of the tree, traceable from the ground to a height of nearly 20 meters before visibility became uncertain.

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[5] The exception was the raid of July 18, which started from the bivouac on the trunkline developed on the previous day and used about 60 meters of that route; however the extension of this raid after mid-morning diverged about 35° from the main direction of July 17. Incidentally, the resumed use of a previous principal trail by a statary colony is not infrequent in *E. hamatum*, although it is relatively rare in burchelli.

Although at first the raids occurred daily and were moderately developed, after five days there were intermittent days without any sign of raiding, or with limited afternoon forays. From the first day at site *B* morning activities began slowly and sluggishly; later on some of the raidless days the ants swarmed down before mid-morning only to withdraw without having begun a foray on the ground. (It is of course possible that unobserved foraging occurred in the upper section of the bivouac tree.) When raids occurred, a general return to the bivouac uniformly began as a persistent movement during the afternoon and was always well under way by dusk. With the exception of ants in procession evacuating the raiding system of the day, no activity of the colony was observed outside the bivouac-tree at night.

When the study had to be discontinued the colony had been at site *B* for 13 days. (Very probably it remained several days longer.) During this time it staged eleven raids, only three of them within the southeast and southwest quadrants.

At this site, as may be seen in Figure 1, the raids were much more concentrated within given sectors than within others. Numerous factors were evidently involved. Downhill at a distance on the south lay a stream nearly impassable to the burchelli, also in beginning a raid the burchelli masses frequently were deflected from that side by the narrow columns of a hamatum colony at the time in statary bivouac near the stream. Uphill toward the north, along a 30° slope, the going in general was easier; then too, the burchelli columns generally reached the ground on that side of the bivouac tree.

The successive raids were well differentiated in direction, with the smallest angle of difference between the lines of successive raids 50°, the largest 165°. Except for an occasional overlapping close to the bivouac, no two successive raids followed the same route, a result not attributable to chance.

Condition of successive broods in colony 381: When it came under observation colony 38I had a single brood (estimated in the tens of thousands) in the advanced larval condition. A thorough examination of the bivouac on June 20 (four days before the colony stopped moving) disclosed no other broods. In the bivouac the larvae were distributed widely throughout the mass; many were heaped in interior pockets, and large numbers were held individually by ants in the outer wall of the cluster. During bivouac-change movements the larvae were carried individually, gripped anteriorly in the carrier's mandibles and slung underneath her body in the typical Eciton manner. In general, larvae were transported in the latter half of the movement.

Although the beginning of cocoons by mature larvae in this brood was first observed on June 23, it may have begun shortly before that time, since three of 40 larvae taken to the laboratory were seen in the act of spinning on June 22. On June 24, when the colony began its long-term residence in the hollow tree at site *A*, spinning was in progress as a general activity. Larvae were then spread about over the detritus flooring the tree hollow, with a constant interpassage of larva-carrying adults between the cluster above and this spinning area below, as described for burchelli by Beebe (1919). The process of forming the outer envelopes appeared to have been finished before June 27, since all larvae that fell to the ground when the bivouac was punctured were enclosed, and all of the laboratory lot of larvae were covered by that time. This does not mean that the larvae had become immobile. Movements of enclosed larvae within their tissue envelopes were observed up to June 28, indicating further spinning through which cocoon walls were thickened. It can be said that colony 381 entered the statary condition rather abruptly on the day when the major part of its larval brood became enclosed.

This brood pupated and passed through its pupal stage while the colony was stationed at site *A*. The removal of callows from cocoons (by workers) began on July 11 and continued on following days, although at first the activity was limited, judging by the fact that only a few dozen empty cases fell from the bivouac on any one day before July 14. Gradually thereafter the removal of callows continued at a sharply increasing rate, indicated not only by a considerable increase in the number of empty cases on the ground and interior wall of the tree below the cluster, but also by a great increase in cocoon-opening visible at the surface of the bivouac. This activity appeared to reach its peak on the night of July 16 and during the day on July 17. On July 17 the colony staged a great raid and moved from the site at the day's end. The bivouac-change column that night was thronged with callow workers. Cocoon-opening evidently was nearly completed before the move, since it was estimated that only a few hundred cocoons were carried to the new site, where the last callows were removed on July 18.

Thus the time when nomadic activity was resumed by this colony coincided exactly with the appearance of a mature brood as newly delivered callows. At first these young workers remained at the bivouac except during bivouac-change movements; however during the first week after leaving site *A* they were observed at points in the raiding system successively farther from the bivouac, and on the sixth day (July 23) they were numerous in the raiding swarm. At that time pigmentation had increased so that they had nearly the color and dusky appearance of adult workers.

A further brood in the early larval stage was found on July 18, when the first nomadic bivouac was examined. In a sample of 82 larvae taken on the evening of July 24, body lengths ranged between 2.4 and 6.3 mm. At that time, therefore, this larval brood was well advanced in growth.[6] Since the colony had only a brood of mature larvae when it came to site *A*, this new brood must have hatched from eggs laid during the stay in the hollow tree. The queen, observed in the latter section of the July 19 bivouac-change column, was fully contracted. Her condition evidently did not change during this period of daily movement, since no eggs were found in periodic examinations of the bivouac and the queen was still fully contracted when seen on the night of July 26.

The new brood, in the early larval condition when the new nomadic period began, advanced through its larval development during the following days of colony movement. On July 25 scattered instances of spinning were observed, and on July 27, with the colony clustered around a small inclined tree well away from the ground, spinning activity was well under way. All circumstances indicated that when the colony settled next day within the hollow tree at site *B*, the bulk of its mature larval population was engaged in spinning or already provided with an outer envelope.

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[6] This may be compared with a sample of 700 larvae taken from another burchelli colony on the first day of a nomadic phase, when body lengths ranged between 0.32 and 1.9 mm. (skewed as usual toward the smaller sizes).

A parallelism between colony behavior and brood condition during the 53 days of this study is clearly indicated in the findings. In particular, it will be noticed that the behavior of the colony and the status of its brood were in substantially the same relationship at the end of the second nomadic period (i.e., on July 27) that they held at the close of the first period (i.e., on June 23). In both cases the colony ceased maximal raiding and bivouacked in a secluded place when its mature larval brood had reached the peak of its relatively brief spinning episode. If the study could have continued about one week after August 10, without much doubt the second statary period (i.e., at site *B*) would have been observed to end as did the first one, with the emergence of a mature brood as callow workers.

It is regrettable that the time when the new batch of eggs was laid could not be ascertained during either statary period. Circumstantially we know that eggs were laid at some time during the stay at site *A*, and unless the statary period at site *B* was very exceptional, we may surmise that the same event occurred there. Direct facts concerning the oviposition were not obtained in either case, since at site *A* the colony was beyond close examination and at *B* was entirely out of reach. Fortunately, observations on other burchelli colonies over shorter periods of time are available which indicate that in the rainy season a batch of eggs is laid early in each statary period, and which fully corroborate the description of a brood-colony behavior parallelism given above.

Observations paralleling the study of colony 38I: On July 18, when colony 38I was beginning an interval of nomadic activity, a burchelli colony was discovered in the southwest section of the Island, sequestered in the crown of a tall palm tree. Although the bivouac cluster itself was never seen, its position was approximated by noting where columns emerged to descend the trunk in the raids of succeeding days. This colony remained on record until August 6. During these 18 days ten raids were observed, probing successively in various directions, all of these raids relatively small and slow to begin in the morning. In the nine days after July 20 there were four days when no raiding occurred, although forays were staged on each of the last six days of the colony's stay in the palm tree. The colony moved to a new site on the evening of August 6, and a further move was observed on the following evening. Thousands of callow workers crowded the columns, most of them very recently emerged as indicated by their limitations of movement and pale pigmentation. Unopened cocoons were transported in the column; however their limited number in comparison with the hordes of callows indicated that the great majority of the mature brood had been removed before the march began. In addition, a very young larval brood was present, carried in several small packets by ants in the column. (In a sample of 50 larvae, the body lengths ranged from 0.3 to 1.8 mm.)

These circumstances suggest that the bivouac-change movement marked the end of a statary period of 18 + days in the palm tree, during which a pupal brood matured and a further brood passed through its early larval development. In addition to the typical occurrence of raidless days, it should be noted that raids occurred on all of the last six days at the site. A fact of interest is that this colony remained in place throughout the 11-day nomadic interval of colony 38I, and took to the march six days after 38I had stopped at site *B*.

A different periodicity was involved in another colony for which the onset of the statary period was observed. On July 24 (i.e., just four days before colony 38I reached site B) this colony was engaged in a large raid about one-fourth mile east of the laboratory clearing. That night it moved about 120 meters to a new bivouac site, and on the following day a large raid gave rise to a move (a short one) to a new site south of the clearing. This time the colony clustered about four meters from the ground, within an exposed hollow in the trunk of a large tree. On the evening of July 25 and on the following day extensive spinning activity was observed, with heavy columns of workers carrying larvae between the bivouac cluster and a debris-strewn place in the cavity near the ground. Here larvae were spread around in the wood-dust and were constantly being shifted in position by workers (cf. Beebe, 1919). On the morning of July 27 a general reduction of activities in and near the bivouac indicated that the major episode of spinning had terminated. The bivouac then assumed a regular pouch-like form which was changed only in minor ways during the following two weeks.

The colony was still bivouacked at this site on August 10, after it had passed 17 days there in the statary condition. During the first week of the stay, there were daily raids of only moderate vigor. Later, in the last ten days of the observation, forays became less active and less frequent than before,—three raidless days were noted, and very probably there were others. Because of the position of the cluster its contents could not be examined closely, hence the appearance of a possible new brood could not be ascertained.

It will be noted that this colony began its statary period just two days before colony 38I stopped at site B, and that both colonies remained in place when the investigation stopped. The externally observable circumstances were much the same in the two cases, especially the concurrence of the final halt with principal enclosure of the mature larval brood and with a marked reduction of raiding from the nomadic level. Numerous studies of shorter duration with other colonies fully confirm these observations.

One important fact should be added here. Among the colonies for which the beginning of the statary period was recorded, in three instances the bivouacs were accessible to direct examination of their contents. From the study of these colonies during the first two weeks of the interval it can be said that a new batch of eggs appears at some time between the sixth and the fourteenth day. Considering the fact that broods of very young larvae at closely comparable stages of development are found in the colonies just beginning a nomadic period, the above observations may be taken to indicate approximately the typical starting time of the new brood in burchelli.

An experimentally modified burchelli routine: On June 17, 1933, a burchelli colony was discovered (on Donato Hill) in a pouch bivouac suspended from crossed vines against the side of a large tree, mainly within a deep recess between buttressed roots and behind large vine stems, about three meters from the ground. The colony remained in this situation until June 26, despite being disturbed on three separate occasions when the bivouac was opened for examination of its contents. At best the raids were only moderately developed, without a major swarm division. During the first five days of the study there were two raidless days, and on another day the foray began in the afternoon. Then in the next five days, just preceding a change to nomadism, daily raids occurred.

When the study opened, the colony possessed a fairly advanced pupal brood, entirely enclosed, and some large packets of eggs were also present. On June 21 and 22 there were indications of cocoon opening; on the afternoon of June 24 removal of callows was actively in progress, and on June 25 when a vigorous raid occurred the removal of callows seemed to reach its peak. That night the colony moved, leaving a litter of pupa cases on the ground below the vacated bivouac site.

However, the colony left the site with only a fraction of its callow brood. On June 23, using ether, I collected an estimated three-fourths of the advanced brood, mainly advanced pupae still enclosed, with a minority of free callows, together with a small part of the larval brood. A subsequent census of the material gives a total of 31,298 individuals in cocoons, all far advanced in pupation.[7]

To find whether there were any polymorphic differences in developmental point attained the cocoons were sorted according to size and then were separated into two groups: one, well pigmented and two, little pigmentation. (Pigmentation here may be taken as a rough indication of nearly complete maturity.) It was found that only 2 per cent of the smallest individuals (median length, 5.0 mm.) had much pigmentation, whereas 50 per cent of the next larger group (6.5 mm.), 89 per cent of the next larger (7.5 mm.) and virtually all individuals in the largest sizes (median lengths, 9.0 and 10.2 mm.) were well pigmented. This shows that the greatest number of pupae furthest advanced in development tends to be in the larger polymorphic categories.

In these facts, together with our observation that ordinarily only well-pigmented individuals (i.e., those capable of movement) are removed from their cases by adult workers, we find in these facts confirmation of Müller's impression that workers major and the larger sizes in general tend to emerge as callows in advance on the smaller individuals. Our observations on the responses of adult workers to the nearly mature brood (1934) show why the larger individuals, those more advanced in development, are the ones to be removed first from their cocoons. After June 23 it was observed that antennal and leg reflexes were common among the best pigmented individuals, and many of these callows survived when artificially removed from their cocoons. Direct observations in the laboratory yielded further evidence that the opening of cocoons by workers depends to a great extent on the responses of adults to the movements of mature pupae within the cocoons, a fact previously reported for *E. hamatum* (Schneirla, 1934).

In the following days the behavior of this colony was exceptional, in that it seemed definitely sluggish and reduced in comparison with an ordinary colony in the interval following the appearance of a callow brood. Throughout the week following the first bivouac-change movement, although daily raids occurred these were inferior in vigor and numbers to maximal burchelli forays, and frequently a daily raid was not followed by a change of bivouac.

On the second nomadic day (June 26) the colony moved, but covered a relatively short distance of 40 meters; on the third day (June 27) there was no colony movement, and although the bivouac was changed on the evening of June 28 this too was over a relatively short distance of 25 meters. On June 29 (when callows were first observed in the swarm), a raid to the west ended in a general return to the bivouac of the day, a cylinder extending to the ground from matted vines. Similarly on June 30 a raid toward the northeast was only moderately developed, and at 4:00 P.M. a general return to the same bivouac was well under way. On July 1 a raid to the southeast ended in a short bivouac change of about 30 meters. However, the move on the evening of July 2 took the ants about 70 meters to the southeast, and that of July 3 eastward about 60 meters where they clustered within

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[7] The results of the census were obtained mainly through the capable assistance of Mr. Frank Trainor, whose services were made available through the Gibson Committee and W.P.A.

TABLE II

Summary of principal changes in the condition and behavior of an *E. burchelli* colony on record from June 17 to July 25, 1933

Period	Bivouac	General nature of raiding	Occurrence of colony movement	Condition of brood
I June 17 to June 26 (statary)	Pouched against side of tree, well above surface of ground.	In general, minimal, with no forays on certain of the days; increasing activity near end of the period.	No bivouac-changes. Returned to same clustering place at end of each day's raiding	Enclosed pupal brood, emerged from cocoons at end of period; three-fourths of this removed. New brood of eggs and young larvae found 6/18.
II June 27 to July 7 (nomadic)	Generally a cylinder hanging from low object to ground; less often a suspended pouch.	Only moderately active at first; maximal raids in latter part of period.	Sluggish during first days, short moves or failure to move; daily and longer movements later.	*Reduced* callow brood emerged at start of period; larval brood passed through principal growth during the period.
III July 8 to July 25	Long pouch hanging from vines, around slender tree, 7 M. from ground.	Largely minimal; on certain days no raiding at all, on others slow start or beginning in afternoon.	No colony movement.	Mature larval brood enclosed at beginning of period, passed through pupation. New brood (eggs, newly hatched larvae) seen on 7/23.

a hollow log. (Examination of the bivouac on the two preceding days disclosed that the larval brood was now moderately grown and fairly well distributed through the cluster.) The raid of July 4 toward the east was quite vigorous, with a major swarm division, and in the afternoon there was a new push from the bivouac toward the south; however, no bivouac-change occurred. On July 5 the raid was vigorous, and that evening the colony moved along this route to a new bivouac site in the distance.

In succeeding days the increased status of activity continued even more markedly than before. July 6 found the colony raiding vigorously to the southwest, with a swarm division in mid-morning and a new foray toward the southwest which developed after 3:00 P.M. The colony moved that evening over the latter route, to form an elevated bivouac hanging in a mass of vines about 130 meters from the previous site. When the cluster was broken open the larval brood was found to be far advanced in development, with some of the largest individuals already enclosed. On July 7 there was a vigorous raid southward, and in the evening a movement occurred to a site about 75 meters distant, where a pouch bivouac was formed against the side of a large tree six meters from the ground. On the following day, July 8, the raid was unusually well developed. At the bivouac site cocoon-spinning was the prevalent activity, with columns of larva-carrying workers much in evidence between the bivouac and a bark-strewn area between tree roots. That evening the colony moved only about 40 meters, to form a pouchlike cluster suspended from a mass of vines seven meters from the ground around a small tree. Since an inspection of the brood during the movement revealed that most of the mature larvae already were enclosed, the beginning of a new statary period appeared to be at hand.

Upon my return to the Island on July 13 from work in another locality, this colony was found as expected, bivouacked in the same place and in nearly the same form in which it had been left on July 8. The records of succeeding days concerning raiding may be briefly summarized as follows: 7/13, a moderately developed raid to the south; 7/14, a moderate raid to the northeast; 7/15, a slowly developed raid to the northwest; 7/16, no raiding; 7/17, a raid to the west, not under way until after 10:30 A.M.; 7/18, a limited raid to the northwest; 7/19, a rather feeble raid to the north, started in the afternoon; 7/20, no raid; 7/21, a relatively small raid toward the north; 7/22, a moderately developed raid to the southeast; 7/23, no raid; 7/24, a moderate raid toward the southeast; 7/25, a moderately developed raid to the south.

When the cluster was opened with a pole on July 23, samples of the brood which fell to the ground were found well advanced in pupation. In addition there were specimens of a new brood, packets containing eggs as well as some newly hatched larvae. On July 25, when my stay at the Island closed, the colony was still bivouacked at the site first occupied on July 8.

During an observation period of 39 days this colony evidently passed through two different statary periods separated by an interval of (modified) nomadic activity. The ten statary days at the outset were regular (i.e., in the usual pattern), with reduced raids in various directions from the site, but without raids on occasional days. Also typical was the presence of an enclosed pupal brood (the appearance of which as callows marked the end of the period) and a new brood of eggs and young larvae. However, on June 23 the major portion of the pupal brood was removed, so that the complement of callows delivered into the colony at the end of the period was only a small part of the number ordinarily appearing.

In the following 12 days the behavior of this colony resembled the pattern already described as nomadic, although at first there were marked differences from the typical course of events. The first raids of the period were relatively small, throughout the first week the bivouac-change movements were shorter than usual and sluggish in their development, and on certain of the days no movement occurred. However in the latter part of the period raiding increased in vigor, and during the last five days bivouac-change became a nightly event. Two facts concerning the brood are notable: in the early part of the period a greatly reduced callow brood was present; and in the latter part of the period a nearly complete larval brood was approaching maturity. The first condition, as suggested above, appears to have been involved in the alteration of initial nomadic events in colony behavior; the latter condition in the appearance of typical nomadic activity during the latter part of the period.

That the relatively brief departure of this colony from the typical burchelli pattern depended upon special and temporary conditions is also shown by circumstances in the statary period last observed. The beginning of statary behavior came, as usual, when the largest part of a mature larval brood became enclosed. It may be surmised that upon the eventual appearance of this substantially unreduced brood as callows, the colony was able to enter a fully vigorous nomadic status.

No great importance attaches to the fact that the two statary bivouacs of this colony were established in exposed places rather than within secluded places such as hollow logs or trees as is typical of burchelli (and hamatum) colonies. In the northeastern section of the Island where the colony operated at the time, the forest is largely second growth in which hollow standing trees or logs are few and well scattered. In the southern and western sections where the timber is heavier, hollow trees and logs are relatively frequent in occurrence, and accordingly statary bivouacs established there by burchelli colonies are rarely exposed.

Evidence for a reduced colony excitement in the statary period: The difference in burchelli raiding during statary and during nomadic times is, on the whole, very striking. Typically, as already described (Schneirla, 1944a), in the nomadic period raiding begins at dawn, a directionalized swarm is set up relatively early, and the extensive development of the foray is indicated both by the great size of the initial swarm and by the later formation of sub-swarms. In contrast, in the statary period morning activities begin sluggishly, directionalized mass activity usually develops slowly, and the swarms are relatively small and seldom reach the point of even a minor division. A comparable difference has been reported for *E. hamatum* (Schneirla, 1938), in which, because of the nature of raiding, the contrast is more strikingly apparent than in burchelli.

However, one nomad-statary difference stands out more prominently in burchelli than in hamatum; namely, a marked reduction of activity outside the bivouac during the middle of the statary period. In four of our longer burchelli records which cover the first two weeks or more of this period, in each instance raiding occurred on every one of the first six days. Then, as in the first and second statary stops of our 38I colony (Table I) and as in Müller's colony, occasional raidless days began to appear. With only minor variations in different instances, in general it can be said that the interval from the seventh to the sixteenth days (roughly) of the statary period is a time of distinctly lowered colony excitement. Frequently then the Ecitons spread out in the morning around the bivouac itself, but in insufficient numbers to form a directionalized swarm, or a raid may not get under way until afternoon. Thereafter, in the terminal days of the statary period, daily raids appear to be the rule.

Through the described interval of reduced external activity, there are reductions in certain internal conditions of the burchelli colony. When a colony enters the statary period its mature larval brood, although enclosed, has not completed its spinning, and the termination of this activity involves an observable stirring of the spinners within their cases during the first few statary days. Although this limited activity of the brood apparently does not affect the workers nearly as much as does the activity of free larvae, laboratory observations suggest that it is a stimulative factor to be reckoned with. During the first few statary days there is a shifting about of cocoons and a stirring of workers holding cocoons which is not observed later, when the cases containing the quiescent pupal brood are held in place by relatively immobile workers over considerable intervals of time. It appears that the reduction of predatory activities typically observed in the intermediate portion of the statary period is due mainly to the virtual disappearance of special stimulation from the brood once it has completed spinning and has pupated.

In the light of the above suggestion, it is significant that raidless days disappear and the forays increase somewhat in vigor during the *last* statary week, the time during which slight movements of the mature post-pupae are observed and when increasing numbers of emerged callows are mixing with workers. That stimulation from the brood becomes a factor of importance is suggested in particular by: an observably greater response of workers to cocoons containing motile individuals than to cocoons containing quiescent brood; a greater activity in experimental groups of workers to which numerous callows have been added than in groups to which equivalent numbers of adult workers are added; and the fact that the first nomadic movement of a colony takes place on the very day the major part of its callows appear.

Function of the burchelli queen: For the extensive study of the Eciton queen I have concentrated upon hamatum, especially because its colonies generally are more accessible to study and their capture less arduous than those of burchelli. What evidence is available on the queen of the latter species indicates that her properties are similar to those already described for hamatum (1944b).

Numerous observations on the condition of broods at different phases of colony activity, involving many colonies, indicate that the burchelli queen must lay a given enormous batch of eggs (30,000 +) within a relatively short time, repeating the process at well separated intervals during the rainy season. Each batch of eggs is laid during a statary period, probably within a time of five or six days near the end of the first third of this period. This is evidenced indirectly by the fact that in all colonies accessible to examination at the time no young have been found in the first six days except an enclosed pupating brood; whereas roughly on the tenth day one begins to find packets of eggs and, later in the period, newly hatched larvae as well. Since under the conditions of this study (i.e., rainy season) the queen of each colony apparently delivers without fail a new batch of eggs early in each statary period, the interval between reproductive episodes must be close to 34 days, i.e., from the early part of one statary period to the corresponding part of the next statary period.

At other times within the statary period excepting the relatively brief physogastric interval, and throughout the nomadic period, the queen evidently remains contracted. Burchelli queens observed on several occasions at various times in the nomadic period always have been contracted, and attendant circumstances in each instance indicate that some time has elapsed since the queen was last gravid. For example, a single queen was taken on July 22, 1933, from a burchelli colony judged to be nomadic from its highly developed raids and from the fact that daily bivouac changes occurred during the three days preceding and the three days following the capture. This queen was fully contracted at the time of capture. The presence of a huge brood of small larvae, all hatched, served to indicate that the colony had emerged only within recent days from a statary period, a conclusion further supported by the presence of many incompletely pigmented callow workers.

Our findings thus oppose the impression of Bruch (1934) and other writers that the contracted Eciton queen is a young individual, still non-functional. The regular spacing of broods which is the rule suggests that a single queen, functional at well separated intervals, is the reproductive agent of each colony. Captures of burchelli and other Eciton queens favor the same conclusion. In the capture mentioned above, only one queen was found, with two broods distinctly different stages of development. The two colonies of *E. burchelli urichi* and *E. burchelli jeanae* which Weber (1941) examined in Trinidad similarly contained but one queen each, in the contracted condition, as did colonies of seven other *Eciton s.str.* species from which queens were captured in various parts of tropical America (Bruch, 1934). In this terrestrial Eciton subgenus a physogastric queen has been taken in only one instance, from an *E. hamatum* colony just ending the first week of a statary period (Schneirla, 1944). From the fact that in the same study 14 queens, all contracted, were taken from as many colonies of that species at various other times in the cycle, the brevity of the queen's delivery interval may be appreciated in contrast to the length of the intermittent resting interval.

An apparent exception was the burchelli colony in Kartabo, British Guiana, from which Wheeler and Emerson (Wheeler, 1921) removed two queens on July 21, 1920. Wheeler judged that both individuals were recently emerged young queens, because of their brilliant coloration as well as that "their ovaries were undeveloped as shown by the relatively small size of the gaster." Although neither of these reasons is very dependable, particularly the latter, the conclusion is made reasonable by the fact that large numbers of enclosed mature male pupae were found in the same colony. If these were both new queens, the possibility remains that the "old queen" of the colony may have escaped unnoticed in the general confusion. Strong and agile, these individuals are difficult to detect and capture unless an anesthetic agent is used. Furthermore, since these captures were made near the end of a rather irregular British Guiana rainy season, the circumstances do not seem comparable to those of our study. In the regular Panama rainy season the broods of burchelli colonies are very large, contain worker forms exclusively, and appear at regular intervals as do those hamatum (Schneirla, 1944), indicating the functioning of a single queen in each colony.

Summary and Discussion

Our evidence indicates that the theoretical conception of an Eciton nomad-statary cycle previously established for *E. hamatum* also holds for burchelli despite marked differences in the behavior pattern of the two species.

Certain differences are apparent in the timing of the cycle in these two terrestrial Eciton species. In *E. hamatum* we have found (1938) that the phases are nearly equal in length, the nomadic interval about 16 to 18 days and the statary interval about 18 to 20 days. In burchelli the nomadic phase appears to be much shorter, approximately 11 to 13 days, and the statary phase considerably longer (21 to 24 days) than in hamatum. The clarification of this species difference in the cycle demands further evidence.

Probably more important is the fact that the total length of the behavior cycle appears to be nearly the same in the two investigated species, approximately 35 days from the beginning of one nomadic phase to the beginning of the next one. Since our evidence indicates that the initiation of each new nomadic phase depends upon influences set into effect with the emergence of a new callow brood, this species similarity in the total cycle length would appear to depend upon an equivalence in the spacing and in the developmental period of the brood in the two species.

The existence of various combinations of regularly concurrent events in the life of burchelli, noted in part by Müller in his study of a single colony in Brazil, is well established by our results. Significant parallels regularly observed in colony behavior and in the intra-colony situation are the following: One, during approximately one week before a colony breaks out of the statary phase, callows in increasing numbers are removed from their cocoons, and on the day of the first nomadic movement it is noted that the bulk of the mature brood has emerged. Two, when nomadism begins a new brood is present, then at an early stage of larval development. Three, as this brood passes through its larval period the colony remains nomadic and becomes increasingly active, but four, when the larvae reach a point of sufficient maturity to permit spinning, the colony stops in place and its raiding activity falls off in vigor on the day the greatest part of the brood becomes enclosed. Five, in the statary phase, once the brood has completed its spinning and is pupating the colony falls to its lowest point in raiding activity, and sluggish raiding prevails until pupae have matured sufficiently to begin stirring within their cocoons.

In many burchelli colonies studied for periods of two days or longer in the Panama rainy season these parallel events in colony behavior and in brood condition have appeared invariably. Without much question a causal relationship is involved.

How do our findings compare with Müller's? His results fall smoothly into our concept of a cycle of events, when we regard the first three days of his record as the end of a nomadic phase and the ensuing days as statary. However, our interpretations differ, particularly it seems, because Müller's facts were incomplete. At the start he was insufficiently impressed by the concurrence of brood enclosure and cessation of colony movement, hence later when the colony remained immobile despite repeated attempts to stir it up, he was led to explain the intertia in terms of a presumed difficulty of moving a queen. Now as an explanation of Eciton colony movement or its absence the queen-transport hypothesis seems quite barren (Schneirla, 1938; 1944b). In a colony movement, the queen generally appears at a time too late to influence the issue of bivouac-change; moreover, she evidently becomes physogastric *after* the colony has become statary, and returns to the contracted condition long before the colony resumes its nightly marches. Actually, colony mobility seems to depend upon *condition of the brood*, a matter which Müller restricted to variations in raiding.

Müller's hypothesis for differences in raiding was essentially teleological. Having noted a decline in raiding when the brood became enclosed and in pupation, he thought "nothing seems more natural than that the food need of the colony is reduced and the ants go out correspondingly less for booty after the larvae are enclosed" (p. 87). Had the study continued to the point of resumed nomadic activity, the concurrent appearance of hordes of voracious callows might well have appeared to furnish additional support for this view. However, a closer examination of this telic food-need hypothesis shows that it merely calls attention to an existing combination of circumstances (i.e., the greatest raiding and greatest mobility of a colony occurring when the brood is active and is feeding), without explaining how these circumstances happen to arise consistently together. It is quite apparent that the pattern of events is one of great adaptive value; with that granted, we contend that the essential causes of the combination lie in the evolutionary background of the species. There food-scarcity factors may well have been involved; however, we find no indication that they are required to make the adaptive pattern effective in the current situation.

For example, a burchelli colony with a maturing larval brood becomes statary (i.e., stops moving; reduces its raiding) on and after the day when this brood has spun its filmy outer envelopes. The total food requirement of the colony must fall considerably with this subtraction of brood feeding, but it is difficult to see how that fact in itself can account for the altered activity pattern of the adult workers. Teleology here tends to obscure and distract attention from the actual causal factors in the given situation. If it is considered too disingenuous to ask how creatures as psychologically limited as are Eciton workers can estimate colony food needs and act accordingly; at least we may inquire how a food-need hypothesis can account for changes in colony raiding *during* the statary period, when the advanced brood is safely enclosed and the new brood (once it appears as eggs) is not feeding.

The facts require a naturalistic, non-teleological explanation. A theory of this nature which seems to fit the situation has been derived from previous work with *E. hamatum* (Schneirla, 1938). Essentially based upon Wheeler's (1928) *trophallaxis* concept, this theory accounts for changes in the overt behavior of an Eciton colony in terms of periodic changes in the total effect of interindividual stimulation developed within the aggregation. The trophallactic conception takes account of the many ways in which workers may be stimulated tactually and chemically by the brood, in handling or carrying about the young, in licking or feeding them, in passing among the brood or in meeting workers that have been aroused by brood relations such as these, and the like. According to this view, the presence of a brood influences adult behavior to the extent that the young, through their activity and effulgence of chemical stimulation, may increase the activity of workers (with consequent summation effects among the latter), so that activities outside the bivouac are increased correspondingly. Thus the critical factor is the amount of incidental stimulation furnished by a brood, whether or not it is consuming food at the time.

Only on this basis can we account for changes in colony behavior in the absence of brood feeding. In particular, during the statary phase raiding falls to a low point after the first week and remains there until the last week, although brood feeding seems absent throughout. It is probable that these changes in colony behavior depend mainly upon differences in tactual stimulation from the brood. At the beginning of the period the brood drops greatly as an excitatory factor when the larvae are first covered with silk, however a partial stimulative effect is furnished by the spinning activities until the cocoons are finished. Thus daily raids occur although the brood has ceased to feed and the colony does not stand in need of food on its account. These raids are inferior to nomadic raids, since larvae stimulate workers more when they move freely and present an open chemostimulative cuticular surface than when their movements are reduced and only the dry, silky envelope surface is presented. Later in the period when the brood is finished with spinning and is pupating, its members quiescent within their cases become passive contents of the bivouac, then, lacking the brood increment in colony "drive" the workers fall to a new low in their raiding. Colony activity continues at this low level until the brood again becomes a functional source of stimulation.

This interpretation may be extended to explain a resurgence of activity in burchelli colonies about one week before the statary period ends. A considerable portion of the pupal brood is then nearly mature, and capable of slight movements (within the cocoons) which observably stimulate the workers. As laboratory observations show, this stimulative effect is exerted *before* the opening of cocoons has begun. The slight tarsal and antennal twitchings and limited dorsoventral contractions of the enclosed callows are visible to the naked eye, and can affect the workers through pressing upon cocoon walls. Workers in an artificial nest containing motile enclosed brood carry the cocoons about and handle them more, and display greater activity in general, than do workers provided with an equal number of enclosed immobile pupae. As a control, mature (pigmented) pupae immobilized by needle puncture of the head do not exert this effect.[8]

The irradiation of such excitement through the bivouac heightens the general responsiveness of the colony, so that a greater reactivity to light causes raids to begin more promptly after dawn than was previously the case, and leads to the impressing of larger numbers into each foray. On the other hand, while the brood remains inactive "colony drive" depends upon the interstimulative relationships of adult workers alone, and must remain at a minimum until a new source of augmented stimulation appears.[9]

In burchelli, as in hamatum (Schneirla, 1938), the appearance of a new callow brood furnishes the impulse for the resumption of nomadic behavior. Then vigorous daily raids occur, each reaching the threshold of bivouac-change movement (Schneirla, 1944a). That this change depends upon the amount of stimulation received from a large callow brood is indicated by our results from a colony deprived of approximately three-fourths of its mature pupal brood late in the statary period. A recognizable nomadism appeared in this colony when the remaining callows emerged; however the daily raids fell below normal standards, and during the first week bivouac-change movements tended to be short in distance or not to occur. Then the colony became more active and its movements attained the normal frequency and vigor. The latter change evidently depended upon a new brood factor.

Although the emergence of a callow brood initiates the nomadic phase of the behavior cycle, this factor apparently does not remain a major source of "drive" for more than a few days. Observations show that callows then behave more like ordinary workers, less excitatory in their effect upon the colony. The above results are what would be expected from a major reduction of the callow-stimulation factor. However, the reinvigoration which occurred in our experimental colony suggests that after about one week of nomadic activity a new excitatory agency becomes effective. Evidently this is introduced through the sufficient growth of a new larval brood whose presence at that time we have found invariable in both burchelli and hamatum (1938). When a colony first takes to the march the individuals of this brood are very small, gathered together in packets for the most part, and (under laboratory observation) low in stimulative effect upon workers. After a few days when the larvae are larger, this brood is found more widely distributed

[8] In both burchelli and hamatum (Schneirla, 1934; 1938) the cocoons are opened and the brood emerges as a result of workers' responses to the stirring of mature individuals. Although adults holding quiescent young stand quietly with their burdens, others clutching dynamized young frequently shift their hold and move about restlessly, thereby causing other workers to catch hold of the cocoons. The excitement increases reciprocally, and in the tugging and regaining of lost holds the envelope is soon torn open. In this operation the arousal of increased movement of the callow both augments the struggle of the workers and incidentally facilitates tearing of the case.

[9] Ecitons are very simple creatures psychologically, unable to "realize" needs, but able only to react directly in simple immediate ways to specific, present stimulation. For example, burchelli colonies regularly stage their raids in the daytime and not at night when these (almost exclusively chemo-tactual) animals could find their prey readily. This condition prevails regardless of existing food requirements in a colony, simply because the photokinetic effect of light is essential to arouse a foray, and in the absence of light Ecitons become lethargic (Schneirla, 1944a). It should be added that the arrival of daylight is least effective upon a colony when intrinsic social stimulation is at its lowest level in the activity cycle.

through the bivouac, and observably excitatory to the workers. This excitatory effect evidently increases in time as the larvae grow, since in the latter part of the nomadic period raiding tends to increase in vigor and numbers of participants. Because of the influence of the more precocious larvae early in the period, the larval factor must overlap that of the callows to some extent as a stimulative factor promoting nomadism, later fully replacing the callows in this rôle. Finally, as in hamatum, when the bulk of the larval brood becomes enclosed and brood stimulation is greatly reduced, the burchelli colony enters a new statary phase.

It is clear from our findings that burchelli colony movements occur in a regular manner, in a predictable series which begins and ends rather abruptly, rather than occurring haphazardly as many writers have supposed. This fact alone would suggest that their basis is not alimentary, dependent upon the supply of food in given areas, as Heape thought (1931). No essential relationship is found between the food supply in given areas and the mobility or immobility of Eciton colonies which would suggest an alimentary basis for the functioning of the *existing* behavior pattern. Instead, the occurrence of the described nomadic and statary activity phases in alternation seems to depend upon the reproductive condition of the colony, and fundamentally upon the reproductive cycle of the queen (Schneirla, 1944b). Since the basic cause of the existing behavior pattern is an intraorganic, gametic factor which functions cyclically, we may say that Eciton behavior conforms to one of the two important criteria generally accepted as essential to migration.

It will be recalled that Heape and others regard migration as a group movement which is reversible and which has its basis in a reproductive process, reserving the term "emigration" for transferral movements which are not regularly reversible and are based on non-gametic processes. Broadly speaking, the latter term would seem applicable to the unitary Eciton bivouac-change movement, in which a colony changes its dwelling place in a single continuous action. Yet this usage takes certain liberties with Heape's meaning of "emigration," since we have found that even the single bivouac-change movement is clearly dependent upon gametically-based conditions in a colony. If the concept does not fit the unitary Eciton movement, it is far less adequate, indeed very inadequate, to denote the series of movements occurring in a given nomadic phase, in which changes of bivouac take place daily in dependence upon a continuous excitation grounded in reproductive processes. For a given nomad-statary cycle, in which a colony moves from one statary bivouac to the next in a regular and predictable manner, the term "migration" would appear appropriate.

By emphasizing gametic factors we do not exclude the possibility that the evolutionary rise of the Eciton behavior pattern may have involved alimentary factors in an important rôle. For example, if an ancestral form ever existed which failed to move at all or undertook very irregular movements while a larval brood was present, an inevitable shortage of food for the growing members of the colony might well have endangered species survival in favor of variant forms. It cannot be denied that alimentary factors may have exerted a critical influence upon the *phyletic* origin of the Eciton behavior patterns.

However, the question of evolutionary background must not be confused with the problem of accounting for the functioning of the existing behavior pattern. In the causation of the existing pattern the food-supply factor plays only a very secondary rôle, in that the quantity of booty found in the early hours of daily raiding influences the direction of the day's foray; however, *booty haul cannot determine whether or not a bivouac-change movement will occur.* Eciton behavior as it functions in existing species presents many features which are adaptive in rather precise and effective ways: in particular, a colony is mobile when its food requirements are greatest, and at rest when they are least; a colony is at rest when the queen is physogastric, thus incidentally shielding her from external hazards, and on the move when she is contracted and able to travel with greater security. These concurrent conditions presumably arose through a long selective process in evolution, and assuredly have great adaptive value both individually and collectively; but we cannot regard them as factors which play a controlling part in the existing behavior pattern. The mechanism for the latter is a dynamic trophallactic process which depends in particular upon given reproductive properties in the queen, and upon given stimulative and reactive properties in brood and workers. The reproductive factor is of paramount importance for the current appearance of the Eciton pattern.

One of the major criteria of migration is satisfied by the fact that Eciton movements occur regularly and periodically on an intraorganic (i.e., reproductive) basis. The second major criterion employed by Heape and generally accepted is that a directionality exists in one phase of migration which is reversed in another phase. Thus salmon characteristically migrate to sea as young individuals, and return upriver as mature adults; many birds of the northern hemisphere move southward in autumn and northward in spring. At first sight Ecitons appear to lack completely this "horizontal" type of directionality. To an extent however their movements exhibit a horizontal directionalization, in that a mechanism already described insures that a colony will not double in its tracks but must take a new route in each successive daily movement. Still this control over direction is a limited one, which cannot prevent changes in the principal course of a given succession of nomadic movements. Moreover, there exists no indication of a reversal of the direction of horizontal progress from one phase of the cycle to the next.

At this point it may be asked what underlies the occurrence of directionality and its reversal in the generally accepted instances of migration. The basis is not very clear in the current evidence, yet perhaps we may say that one direction of progress in a migration is an expression of given responses to external conditions, and that in the opposite phase of the cycle these responses are changed or reversed, in response to changed or reversed external conditions. The different responses to external conditions in the two phases of the cycle may be regarded as dependent upon different (perhaps reversed) intraorganic conditions.

This interpretation suggests to us one characteristic of the Eciton pattern which may be regarded as the biological equivalent of a direction reversal in the usual sense. We have noticed that during a nomadic phase burchelli (and hamatum) colonies tend to bivouac next the surface of the ground, that toward the end of this phase the colonies tend to cluster at a distance from the ground, and that statary bivouacs almost invariably are formed in elevated positions. This difference, together with the fact that nomadic bivouacs generally are exposed whereas statary bivouacs tend to be secluded, suggests that colonies of terrestrial Eciton species respond differently to environmental conditions according to the given phase of their activity cycle. The outcome may be considered an instance of "altitudinal" migration, somewhat comparable to animals (e.g., some birds) which migrate from valley to hill and return in a different phase of their cycle without any particular reversal of the latitudinal direction taken in the ascent. Apparently the limited ascent and descent during different stages of migration in terrestrial Ecitons somehow has its basis in changed intra-colony conditions centering around the brood. At present both the nature of this basis and of the external conditions involved in the altitudinal movement are matters of conjecture.

The writer believes that a general theory of animal migration cannot be worked out without careful attention to patterns which conform only partially to the traditional vertebrate examples. Thus Williams (1943) and Fraenkel (1932) have presented convincing reasons for including in the general evidence the movements of certain insects such as many lepidopteran and orthopteran species. The Ecitons, it would appear, must also be included. These insects seem to represent an example of primitive or rudimentary migration in which the basis of the pattern is present without a highly specialized superstructure like that found in animals such as many birds, permitting a directionalization of horizontal locomotion. On this view, the organic "drive" basis of migration is fundamental, but may exist in the partial or complete absence of specialized factors permitting a directionalized and reversible movement.[10]

It should be emphasized that the army ants are exceptional among migrating animals in that their movements are carried out by individuals which are not themselves responsible for the origination of the basic drive factor. Indeed the actual process of locomotion is brought about in the workers in a very indirect manner, which involves not only an incidental stimulative function of the brood as intermediate process, but also effects itself through a worker activity (raiding) which is qualitatively different from the true colony movement. As we have seen (Schneirla, 1944a), the very different patterns of swarm-raiding and column-raiding may both lead into migration provided that other factors are involved which are basically independent of the workers themselves. Yet these other factors, the fundamental contribution of the queen and the intermediate rôle of the brood, are smoothly interrelated with the "effector" function of the workers in a highly stereotyped manner, so long as the optimal environment furnished by regular rainy-season conditions exists. What differences may appear in the pattern under the non-optimal conditions of dry-season weather, or in an environment of irregular annual rains, is a problem for further investigation.

CONCLUSIONS

1. The cyclical pattern of nomadic and statary behavior described for the swarm-raiding species *E. burchelli*, and previously reported for the column-raiding *E.*

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[10] Woodbury (1941), in advancing his *periodic-response theory* for migration in general, points out that the visceral rhythms which are held basic to migration may be correlated with environmental periodicities in a variety of ways and to different extents in different animals. In the Ecitons, although seasonal differences in migatory activities apparently exist, and although the day-night periodicity is involved in the arousal of separate movements, evidence is lacking that the timing of the nomad-statary cycle itself is dependent upon extraorganic periodicities. However, for the queen's reproductive cycle which the present writer (1944) has found basic, Weber (1943) suggests that the lunar cycle may play a surrogate rôle. A number of circumstances oppose this possibility in the investigated species, in particular the fact that at a given time colonies may vary considerably in the phases of their brood and activity cycles, also the fact that the duration of the queen's ovulation cycle falls close to 35 days rather than approximating 28 days as the lunar-cycle hypothesis seems to demand.

hamatum, may be considered typical of terrestrial Eciton species. Species differences exist in the relative length of the phases of this activity cycle.

2. The basis of the pattern in burchelli, as in hamatum, is in the rhythmic reproductive function of the colony queen. This factor expresses itself through periodic changes in the stimulative (i.e., trophallactic) effect of broods upon the workers, and thus indirectly controls the presence or absence of regular daily colony movements.

3. Since these Eciton movements occur in a regular and periodic manner, on an intraorganic (reproductive) basis, they may be considered a "primitive" or rudimentary instance of migration. In the army ants there exists a biological equivalent of directionalization of migration and its reversal, in the form of an altitudinal shifting of the home site in different phases of the activity cycle.

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THE RESISTANCE AND ACCLIMATIZATION OF MARINE FISHES TO TEMPERATURE CHANGES. II. EXPERIMENTS WITH FUNDULUS AND ATHERINOPS[1]

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INTRODUCTION

In an earlier paper (Doudoroff, 1942) a detailed study of the resistance and acclimatization of the marine fish *Girella nigricans* to extreme high and low temperatures was presented. Some parallel experiments on the temperature tolerance of the relatively hardy species *Fundulus parvipinnis* and of the very delicate *Atherinops affinis* are reported here. While no attempt has been made to investigate fully the causes of death on chilling, some observations are recorded which bear on this problem.

The literature on the resistance and acclimatization of fishes and of other animals to low and high temperatures has been reviewed in the earlier publication. Of papers which have come more recently to the author's attention, those of Brett (1941, 1944) and of Fry, Brett and Clawson (1942) on the lethal limits of temperature for various fresh-water fishes must be mentioned. The importance of acclimatization as a factor to be considered in delimiting the temperature tolerance of these fishes was demonstrated fully. Certain early assumptions relative to the mortality rate at lethal temperatures (namely, that death occurs almost always within 12 or 14 hours even at low temperatures) and relative to the rate of acclimatization to low temperatures were not fully substantiated and are not entirely in accord with the author's observations on marine fishes.

The survival of some hardy fishes at very low, sub-zero temperatures in a state of seemingly suspended animation formerly had received more attention than death at low temperatures well above 0° C., and frequently has been termed "anabiosis" (Borodin, 1934; Schmidt, Platonov and Person, 1936). This term is misleading. The seemingly lifeless condition of supercooled and superficially frozen fish may be due to reversible injury to the central nervous system. The sensitive automatic mechanism of the heart also may be affected (Battle, 1926). There is no evidence that tissue respiration and all other vital functions are arrested (see also Kalabuchow, 1935), nor that the so-called anabiotic state can persist for a very long time. Therefore, this state may not differ fundamentally from chill-coma at above-zero temperatures and even may be closely related to heat-coma. The low metabolic rate at the lower temperatures and the hardiness of the species in which "anabiosis" has been observed account for the fact that the coma, with attendant respiratory and possibly circulatory disturbances, can be more prolonged at sub-zero temperatures.

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[2] Now with the Louisiana State Department of Wildlife and Fisheries, University, Louisiana.

Weigmann (1936) has contended that the death of fish which he super-cooled to very low temperatures was caused by respiratory disturbances. These he believed to be due to a direct effect of cooling upon the respiratory center of the brain, although other possibilities also were suggested. The observation that the animals could be revived, after return to normal temperatures, by the administration of artificial respiration was advanced as one of several arguments in support of the opinion that some respiratory disturbance was the cause of death. It will be shown that other factors can play an important role in the causation of death by chilling.

The helpful advice and assistance received from Dr. F. B. Sumner and other members of the staff of the Scripps Institution of Oceanography in the course of this investigation are most gratefully acknowledged.

Methods and Material

The methods and equipment used in the evaluation of temperature tolerance were those employed in the experiments with Girella (Doudoroff, 1942). Briefly, the measures of relative tolerance adopted are the lower and upper median tolerance limits (T_m), defined as the low and high temperatures, respectively, which are lethal to just 50 per cent of the experimental animals in a given specified period of exposure. These values are estimated, when necessary, by interpolation, being then based on observed percentages of specimens surviving at two successive, constant test temperatures, usually 1° C. apart, one of which is lethal to more than half, and the other to less than half of the animals tested. Estimation of median tolerance limits for a series of time intervals permits verification of the individual values and frequently evaluation of the ultimate median tolerance limit, that is, the temperature which is tolerated indefinitely by 50 per cent of the animals.

Usually the fish were acclimatized and tested in constant-temperature aquaria supplied with running sea water, but in experiments in which the salinity of the medium was varied, jars with standing, continuously aerated water were used. Unless otherwise denoted here, the medium was sea water.

Fundulus parvipinnis Girard inhabits shallow and enclosed bays, sloughs and estuaries. Adults of both sexes, taken in Mission Bay near San Diego, were used. Average and minimal temperatures in the habitat of the species in this locality probably do not differ greatly from those in open water at the Scripps Institution of Oceanography nearby (Doudoroff, 1942). Maximal temperatures to which Fundulus may be exposed for brief periods in summer are considerably higher, however, than those recorded in open water, temperatures of 30° C. or higher probably being not uncommon during low tide in some places which are frequented by these active fishes.

Young *Atherinops affinis* (Ayres) were taken near the Scripps Institution at La Jolla, where, like Girella, they are common in open water near the shore. This species also is common in bays and estuaries, but, unlike Fundulus, it apparently does not frequent very shallow inlets and sloughs of the coastal marshes, where temperatures much higher than those in open water occur frequently. Thus, Atherinops probably lives under approximately the same temperature conditions as Girella in the same general locality. According to Jordan, Evermann and Clark (1930), the range of distribution of Atherinops extends along the "coast of California and north to Oregon," *i.e.*, further north than that of Girella, while that of Fundulus extends "from Point Conception" (actually it extends somewhat further north) "southward to Lower California."

Comparative tests showed clearly that Atherinops is less resistant and Fundulus is more resistant than Girella both to reduced salinity (dilution) of the medium and to asphyxiation (in tightly stoppered bottles in which the three forms were confined together). In each of these tests, which were performed with groups of specimens of equal average weight, all specimens of each of the less resistant species succumbed long before any of the specimens of the next more resistant species.

Only a relatively small number of the delicate and active Atherinops could be brought to the laboratory and kept for long periods in good health. Fundulus, although hardy, is subject to disastrous epidemic diseases in the laboratory (Wells and ZoBell, 1934) and often is heavily parasitized. Care was taken to select for experimentation only healthy specimens without parasitic isopods on the gills.

RESULTS

Effects of extreme temperatures

Fundulus and Atherinops were affected by exposure to extreme high and low temperatures much as were Girella (Doudoroff, 1942), but the following important differences were noted.

If returned to a normal temperature within a few minutes after the onset of heat-coma, Fundulus, unlike Girella and Atherinops, often showed partial, temporary recovery, and sometimes complete recovery.[3] The administration of artificial respiration, by passing a stream of water through the mouth and over the gills, favored the recovery.

At slowly lethal temperatures primary chill-coma, that is, temporary cessation of all movements almost immediately following transfer to a low temperature, often was more prolonged in Fundulus than in Girella. Fundulus sometimes showed spontaneous, temporary recovery after remaining in a state of complete primary coma for more than one hour.[4] Specimens which failed to resume respiratory movements within two hours after cooling never moved thereafter at the low temperature. If returned to a normal temperature, these fishes were able to recover after longer periods, but recovery after three hours was observed only in one instance. However, recovery was obtained by the administration of artificial respiration together with warming when warming alone was not sufficient. This is illustrated by the following experimental results. Fourteen specimens, which had been held at 20° C., were exposed to 2° C., at which temperature none recovered from primary coma. After three hours the fishes were returned to 20° C., and artificial respiration was administered to one half of the specimens. All of the latter specimens resumed respiratory movements, and all but one recovered completely and were alive after three days. The seven specimens to which artificial respiration was not administered showed no recovery whatsoever.

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[3] Recovery has been obtained with other hardy fishes, e.g., *Gillichthys mirabilis* (Sumner and Doudoroff, 1938). Injury to the integument and paralysis of body muscles sometimes were noted in Fundulus which had recoverd partially after exposure to very extreme high temperatures. At these temperatures bleeding of the gills was observed occasionally.

[4] Such temporary recovery after prolonged primary chill-coma was observed in tests with other hardy species, e.g., *Leptocottus armatus* Girard.

If spontaneous recovery from primary chill-coma occurred at the low temperature at which the coma was produced, Fundulus always survived at this temperature for more than 12 hours before the onset of permanent, secondary chill-coma. For example, of 24 specimens which were transferred from 20° to 3° in a preliminary test only 14 recovered from primary coma, yet all of these survived for 12 hours, and 9 were still alive after 24 hours. The surviving specimens righted themselves and appeared normal. Later, however, they became seemingly stiff and emaciated, their eyes becoming deeply sunken. This appearance suggested osmotic dehydration of the tissues. Secondary chill-coma followed. If warmed thereafter, Fundulus sometimes showed brief activity, but complete recovery was unusual.

In Atherinops temporary recovery after complete primary chill-coma was observed rarely. Violent paroxysms, loss of equilibrium and partial inactivation often occurred after cooling and could be followed by recovery, but if movements ceased entirely for more than a few minutes, the coma was always permanent, and after one hour this species never recovered even on transfer to a normal temperature.

Permanent cessation of respiratory and other movements, either spontaneous or induced by mechanical stimulation, was regarded as the onset of death or end-point of survival, as in the experiments with Girella. With Atherinops, as with Girella, determinations could be made of lower median tolerance limits for exposure periods of one hour or more. Inasmuch as Fundulus sometimes recovered from primary chill-coma after periods longer than one hour, the shortest exposure period adopted for cold-tolerance tests with this species was three hours.

Time-temperature relation of cold-tolerance in Fundulus

Fundulus collected in late summer and stocked at current sea water temperatures of 19° to 21° were soon transferred to constant-temperature tanks at 14°, 20° and 28°. Many of the specimens held at 14° and most of those at 28° died of disease. The relatively healthy 20°-conditioned fish were used in cold-tolerance tests after a sojourn of 30 days or longer in the constant-temperature tank. The experimental results are presented in Table I. Since all specimens which survived for 3 hours survived also for 12 hours, the data for these exposure periods appear in a single column.

TABLE I

Per cent survival of 20°-conditioned Fundulus at various low temperatures and the estimated lower median tolerance limits (T_m) in relation to time (duration) of exposure to the test temperatures

Date 1940	No. of fish and ratio ♂: ♀	Mean length (cm.)	Test temp. (° C.)	Per cent surviving after							
				3 to 12 hours	1 day	2 days	3 days	4 days	5 days	6 days	7 days
8/28	12 (9:3)	6.8	7°	100%	100%	100%	100%	100%	100%	100%	100%
9/4	14 (10:4)	6.7	6°	100	100	100	100	100	93	86	86
9/11	12 (9:3)	6.7	5°	100	100	100	92	67	25	0	0
9/22	12 (8:4)	6.7	4°	100	100	100	83	25	0	0	0
9/18	16 (12:4)	6.7	3°	87	81	50	0	0	0	0	0
9/27	14 (10:4)	6.7	2°	0	0	0	0	0	0	0	0
		T_m:		2.6°	2.6°	3.0°	3.6°	4.6°	5.4°	5.6°	5.6°

The lower median tolerance limits are plotted against exposure time in Figure 1. The sigmoid time-temperature curve of cold-tolerance shown differs strikingly from curves obtained with Girella (Doudoroff, 1942, Fig. 1), which showed most of the rise of T_m with increasing time of exposure occurring during the first day or two. The ultimate lower median tolerance limit for the Fundulus apparently is close to 5.6°.

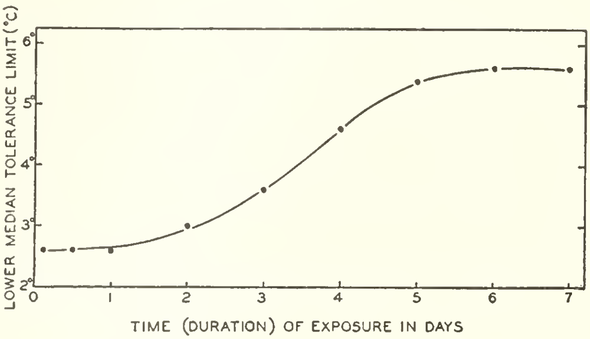

FIGURE 1. Time-temperature curve of cold-tolerance of 20°-conditioned Fundulus: Lower median tolerance limits in relation to the time (duration) of exposure to the test temperatures in sea water. Data from Table I.

Comparison of the data with the results of some earlier preliminary experiments and of later tests revealed a small progressive increase of the resistance of the 20°-conditioned Fundulus to cold, and especially to the less extreme low temperatures. The cause has not been established, but the change may have been seasonal or related to the sexual cycle.

Changes of water content at lethal low temperatures and the influence of dilution of the medium and acclimatization on cold-resistance in Fundulus

The loss of tissue water through osmosis by Fundulus which were dying in sea water at slowly lethal low temperatures was inferred from the appearance of the animals. Data presented in Table II, which shows the relative water content (*i.e.,* weight lost on desiccation to constant weight at 100° per unit of the dry weight) for groups of specimens differing in their history, support this inference. The experimental animals which had been exposed to low test temperatures were removed for weighing when they were seemingly near death, but still respiring and responding to stimulation. The results obtained with 14°-conditioned specimens indicate an average loss of more than 23 per cent of the normal water content at the lethal low temperatures (1.0°–1.6°) in sea water, but no loss in diluted (45 per cent) sea water. The specimens tested in the diluted water did not appear dehydrated and were still quite lively when they were removed, although they had been exposed to the low temperature (1.6°) about twice as long as the specimens tested in sea water. The relatively large 20°-conditioned specimens apparently lost, on the average, 14 per cent of their initial water content at the lethal test temperature (3.0°) in sea water.

Osmotic work must be done constantly by marine fishes in their normal medium. Dehydration can result from disturbances of the osmoregulative functions, but

TABLE II

Water content of Fundulus after exposure to slowly lethal low temperatures in sea water (SW) and in diluted sea water (45% SW), and that of control specimens taken directly from sea water at the initial acclimatization temperatures. All of the experimental specimens were still alive when removed from the test aquaria. The water content is expressed as units of weight of water per unit of dry weight. The mean values indicated for fish with similar history are weighted according to the number of specimens.

Acclim. temp. (° C.)	Test temp. (° C.)	Days at test temp.	Test medium	Number and sex of fish	Wet weight (grams)	Dry weight (grams)	$\frac{\text{Wet wt.} - \text{Dry wt.}}{\text{Dry wt.}}$
14°	1.0°	2	SW	1 ♂	3.64	1.03	2.53
14°	1.6°	2	SW	1 ♂	5.06	1.48	2.42
14°	1.6°	2	SW	1 ♂	4.51	1.38	2.27
14°	1.6°	2	SW	2 ♂	7.55	2.25	2.36
					Weighted mean:		2.39
14°	1.6°	3½	45% SW	2 ♂	9.84	2.45	3.02
14°	1.6°	4	45% SW	5 ♂	19.29	4.57	3.22
					Weighted mean:		3.16
14°		Control		1 ♂	3.51	0.86	3.08
14°		Control		1 ♂	3.93	0.95	3.14
14°		Control		1 ♂	4.06	0.97	3.19
14°		Control		2 ♂	9.91	2.43	3.08
					Weighted mean:		3.11
20°	3.0°	1½	SW	1 ♀	6.46	1.75	2.69
20°	3.0°	2½	SW	1 ♂, 1 ♀	13.63	4.02	2.39
20°	3.0°	3	SW	2 ♂	14.65	4.32	2.39
					Weighted mean:		2.45
20°		Control		1 ♂	8.62	2.13	3.05
20°		Control		1 ♂, 1 ♀	15.61	4.08	2.83
20°		Control		1 ♂, 1 ♀	16.04	4.25	2.77
20°		Control		1 ♂, 1 ♀	11.60	3.01	2.85
					Weighted mean:		2.85

obviously can be prevented by sufficient dilution of the medium. *Fundulus parvipinnis* probably occurs only in salt water, but it can live in fresh water. The normal osmotic pressure of the blood of this species was not known. From available cryoscopic data for other marine and euryhaline fishes (Dakin, 1912, 1935) it was concluded, however, that 45 per cent sea water probably is roughly isosmotic with the blood of Fundulus, or only slightly hypertonic. The results of comparable tests of the cold-resistance of specimens acclimatized to 20° and to 14° and tested in sea water and in 45 per cent sea water (prepared by dilution with water distilled in glass) are presented in Table III. The specimens tested in the diluted water were held in this medium for one day before the test.

TABLE III

Per cent survival of Fundulus at various low test temperatures and the estimated lower median tolerance limits (T_m) in relation to temperature of previous acclimatization, to dilution of the medium (SW = sea water) and to time (duration) of exposure to the test temperatures

Date 1940	Acclim. temp. (° C.)	Test medium	No. of fish and ratio ♂: ♀	Mean length (cm.)	Test temp. (° C.)	Per cent surviving after											
						3 to 12 hours	1 day	2 days	3 days	4 days	5 days	6 days	7 days	8 days	9 days	10 days	11 days
10/6	20°	SW	8 (5:3)	6.7	4.0°	100%	100%	100%	100%	87%	37%	0%	0%	0%	0%	0%	0%
10/18	20°	SW	8 (4:4)	6.7	3.0°	100	100	100	87	0	0	0	0	0	0	0	0
10/30	20°	SW	8 (5:3)	6.7	2.0°	37	37	12	0	0	0	0	0	0	0	0	0
				T_m:		2.2°	2.2°	2.4°	2.6°	3.6°							
10/6	20°	45% SW	8 (5:3)	6.7	4.0°	100%	100%	100%	100%	100%	100%	100%	75%	62%	37%	12%	0%
10/18	20°	45% SW	8 (4:4)	6.7	3.0°	100	100	100	100	100	100	100	37	12	0	0	0
10/30	20°	45% SW	8 (5:3)	6.7	2.0°	37	37	25	12	0	0	0	0	0	0	0	0
				T_m:		2.2°	2.2°	2.3°	2.4°	2.5°	2.5°	2.5°	3.3°	3.8°			
11/12	14°	SW	8 (5:3)	6.7	1.6°	100%	100%	87%	0%	0%							
11/4	14°	SW	8 (5:3)	6.7	1.0°	87	75	25	12	0							
11/8	14°	SW	8 (5:3)	6.7	0.4°	12	12	0	0	0							
				T_m:		0.7°	0.8°	1.2°									
11/12	14°	45% SW	8 (5:3)	6.7	1.6°	100%	100%	100%	100%	75%	? Test discontinued.						

At the rapidly lethal temperature of 2° there was no signficant difference between the mortality rates of 20°-conditioned specimens in the two media, five of the eight fish in each group having failed to recover from primary chill-coma. At the less extreme low temperatures of 3° and 4°, on the other hand, all the specimens succumbed in sea water before any succumbed in the diluted water. If the mid-point of the 24-hour interval during which each specimen succumbed is taken as the time of its death, the mean survival times at the latter two temperatures are found to be 3.4 days and 4.75 days, respectively, in sea water, while in the diluted water they are 7.0 days and 8.4 days, respectively. The difference between the means for the two media at each of these slowly lethal temperatures is certainly significant, the probability being $0.9999+$.[5] Accordingly, T_m values for moderately long exposure periods only were clearly influenced by the salinity of the water. The results obtained with the 14°-conditioned specimens, which had been held at 14° for 83 to 91 days, likewise indicate clearly an influence of water salinity upon the mortality rate at a slowly lethal low temperature (1.6°), and also an influence of acclimatization upon cold-tolerance.[6]

Resistance and acclimatization of Fundulus to heat

Fundulus used in the heat-tolerance experiments were acclimatized in the three constant-temperature tanks (at 20° for 30 to 51 days, at 28° for 57 to 63 days, and

TABLE IV

Per cent survival of Fundulus at various high temperatures and the estimated upper median tolerance limits (T_m) in relation to temperature of previous acclimatization and to time (duration) of exposure to the test temperatures

Date 1940	Acclim. temp. (° C.)	No. of fish and ratio ♂: ♀	Mean length (cm)	Test temp. (° C.)	Per cent surviving after										
					0.5 hr.	1 hr.	3 hrs.	6 hrs.	12 hrs.	24 hrs.	48 hrs.	72 hrs.	96 hrs.	120 hrs.	144 hrs.
9/30	28°	12 (5:7)	6.8	40°	33%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
9/30	28°	13 (5:8)	6.8	39°	100	69	0	0	0	0	0	0	0	0	0
9/27	28°	13 (5:8)	6.7	38°	100	100	100	31	0	0	0	0	0	0	0
9/25	28°	12 (5:7)	6.7	37°	100	100	100	100	100	42	0	0	0	0	0
10/1	28°	13 (5:8)	6.8	36°	100	100	100	100	100	100	100	100	100	100	92
			T_m:		39.7°	39.3°	38.5°	37.7°	37.5°	36.9°	36.5°	36.5°	36.5°	36.5°	36.5°
9/18	20°	12 (8:4)	6.7	37°	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
9/14	20°	13 (9:4)	6.7	36°	54	54	38	38	23	8	0	0	0	0	0
9/4	20°	13 (9:4)	6.6	35°	100	100	100	100	100	100	85	54	38	31	31
8/28	20°	13 (9:4)	6.6	34°	100	100	100	100	100	100	100	100	92	85	62
			T_m:		36.1°	36.1°	35.8°	35.8°	35.7°	35.5°	35.4°	35.1°	34.8°	34.6°	34.4°
10/10	14°	12 (6:6)	6.7	34°	42%	25%	0%	0%	0%	0%	0%	0%	0%	0%	0%
10/11	14°	12 (6:6)	6.7	33°	100	100	33	8	8	8	8	8	8	8	8
10/22	14°	13 (7:6)	6.7	32°	100	100	92	77	77	69	69	69	69	69	69
			T_m:		33.9°	33.7°	32.7°	32.4°	32.4°	32.3°	32.3°	32.3°	32.3°	32.3°	32.3°

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[5] For the method of computing probabilities see Tippett (1931).

[6] Fundulus which had been acclimatized to 28° and then held for 7 days at 36° died within 24 hours after subsequent cooling to 15°. Similar observations have been recorded by Wells (1935).

at 14° for 58 to 70 days) together with those used in the cold-tolerance tests. The experimental data are presented in Table IV.

The time-temperature relationship of heat-tolerance of 28°-conditioned Fundulus is somewhat similar to that observed with 20°-conditioned Girella, the values of T_m for exposure periods of 0.5 to 48 hours tending to fall along a straight line when plotted against the logarithm of the exposure time (Fig. 2). The 48-hour T_m, or 36.5°, apparently is also the ultimate median tolerance limit. The relationship obtained with 14°-conditioned Fundulus is not logarithmic and is not unlike that obtained with 12°-conditioned Girella. All deaths occurred during the first day of exposure, T_m remaining thereafter at 32.3°. The nearly straight-line relationship between T_m and duration of exposure obtained with Fundulus from the intermediate temperature of 20° is quite unlike any others which have been studied. No explanation can be suggested. Nevertheless, the influence of acclimatization upon resistance to heat is shown clearly by the data, and it is evident that Fundulus, unlike Girella, does not acquire nearly maximal heat-tolerance when it is acclimatized to 20°.

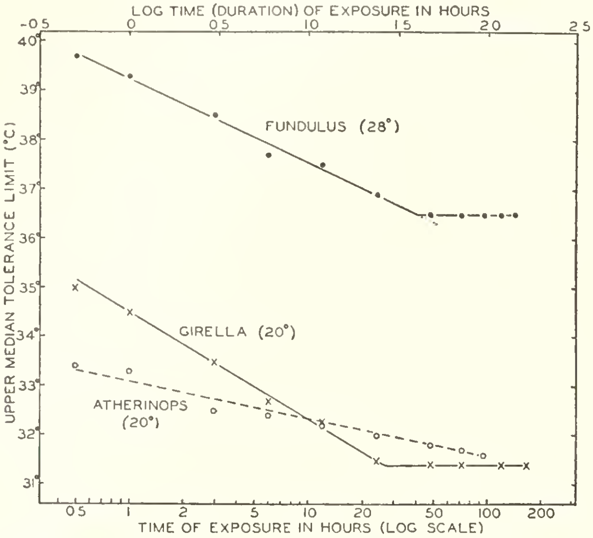

FIGURE 2. Time-temperature curves of heat-tolerance of 28°-conditioned Fundulus, 20°-conditioned Atherinops and 20°-conditioned Girella: Upper median tolerance limits in relation to the logarithm of the time (duration) of exposure to the test temperatures. Data from Tables IV and V and from Doudoroff (1942), Table II.

Resistance and acclimatization of Atherinops to heat and cold

Atherinops taken in October, when the surface temperature in their habitat had been close to 20° for some time, were held in the laboratory for three weeks at current sea water temperatures of 19° to 20°, and then in a constant-temperature tank at 20° for one week before tests were begun. The time-temperature relations of tolerance to heat and to cold were studied in the usual manner, but intervals of only 0.5° between successive test temperatures were used in the evaluation of heat-tolerance to ensure desired accuracy. The results are presented in Table V.

Great sensitiveness of this species to cooling is evident. While no simple time-

TABLE V

Per cent survival of 20°-conditioned Atherinops at various high and low temperatures and the estimated upper and lower median tolerance limits (T_m) in relation to time (duration) of exposure to the test temperatures

Date 1940	No. of fish	Mean length (cm.)	Test temp. (° C.)	Per cent surviving after								
				0.5 hr.	1 hr.	3 hrs.	6 hrs.	12 hrs.	24 hrs.	48 hrs.	72 hrs.	96 hrs.
11/6	12	6.1	33.5°	42%	17%	0%	0%	0%	0%	0%	0%	0%
11/5	14	6.1	33.0°	86	86	0	0	0	0	0	0	0
11/7	12	6.2	32.5°	100	100	50	33	0	0	0	0	0
11/2	12	6.2	32.0°	100	100	100	100	92	50	17	0	0
11/8	14	6.1	31.5°	100	100	100	100	100	100	86	71	57
		T_m (upper):		33.4°	33.3°	32.5°	32.4°	32.2°	32.0°	31.8°	31.7°	31.6°
11/28	18	6.2	11.0°		100%	100%	100%	100%	100%	100%	100%	100%
11/20	18	6.0	10.0°		100	100	100	94	94	44	22	22
11/16	16	6.1	9.0°		100	100	100	94	75	37	6	0
11/15	16	6.0	8.0°		100	75	25	0	0	0	0	0
11/14	16	6.1	7.0°		87	62	0	0	0	0	0	0
11/17	18	6.1	6.0°		56	28	0	0	0	0	0	0
11/16	16	6.2	5.0°		0	0	0	0	0	0	0	0
		T_m (lower):			5.9°	6.6°	8.3°	8.5°	8.7°	10.1°	10.4°	10.4°

temperature curve of cold-tolerance fits the data closely, the lower median tolerance limit rises progressively with increasing time of exposure for three days, whereupon an apparently constant value of 10.4° is reached. The upper median tolerance limits are plotted against the logarithm of the time of exposure in Figure 2, together with corresponding data for 20°-conditioned Girella and 28°-conditioned Fundulus.

Some Atherinops were held through the winter at current sea water temperatures, which were below 18°, and which averaged 16° during the last month. After subsequent acclimatization for about three weeks to a constant temperature of 18.5° these specimens were found to have lower and upper 24-hour median tolerance limits of 8.8° and 30.5°, respectively. While all the fish died within 24 hours after direct transfer to 31° in this experiment, after a two day sojourn at 30° all survived for 48 hours at 31°, and the majority survived at 32°, thus showing rapid acclimatization to heat. Rapid acclimatization to cold did not occur, but in another experiment with specimens which had been held at 20° and then acclimatized for more than two months to 14.5° and 25.5° a pronounced influence of long acclimatization upon cold-tolerance was demonstrated, the 24-hour lower median tolerance limits for the two lots being 7.6° and 13.5°, respectively.

DISCUSSION

Because of the nature and variability of the time-temperature relations of tolerance, prolonged tests often may be necessary in ecological studies of the resistance of fishes to cold and heat. The lower median tolerance limit for Fundulus, unlike those for Girella and Atherinops, remained nearly constant during the first 48 hours of exposure (6 days in diluted sea water) and rose rapidly thereafter (Fig. 1). Limits of tolerance for uniform exposure periods even longer than 48 hours evidently may not be truly comparable and ecologically significant. Had extended observations not been made, the prolonged temporary recovery of Fundulus from primary chill-coma at slowly lethal low temperatures could have been mistaken for permanent recovery.

The time-temperature curves of heat-tolerance for 20°-conditioned Girella, 28°-conditioned Fundulus and 20°-conditioned Atherinops (Fig. 2) are somewhat similar, but they differ greatly with respect to their slope, the temperature coefficients, Q_{10}, of the velocity of heat injury in these fishes being about 4×10^4, 10^6 and 10^{13}, respectively.[7] In each of these instances Q_{10} is constant over the range of lethal temperatures used, but this coefficient evidently is not generally applicable. Bělehrádek (1935) listed temperature coefficients of heat injury for various organisms and tissues, the values of Q_{10} listed ranging from less than 10 to 58,000. Rapid acclimatization probably accounts for the unusually high values obtained with Fundulus and Atherinops.

The ranges of temperatures which were tolerated for three days by average 20°-conditioned specimens of the three species studied are 10.4° to 31.7° (21.3° range) for Atherinops, 8° to 31.4° (23.4° range) for Girella and 3.6° to 35.1° (31.5° range) for Fundulus. The 6-day tolerance limits for the Fundulus, which are 5.6° and 34.4° (28.8° range), are more nearly comparable with the 3-day values for the other two species. The magnitudes of the ranges are clearly related to the general hardiness of the species, and resistance to cold shows a parallel relationship.

The 20°-conditioned Atherinops were about as resistant to heat as Girella, but were more sensitive to cooling, their 72-hour lower median tolerance limit being only 1.3° below the lowest weekly average surface temperature recorded near the place of their capture.[8] While the temperature of 20° lies about half way between the extremes of temperature which may be encountered by Atherinops in this general locality, and is above the average surface temperature, the specimens taken from 20° withstood for four days a rise of temperature of 11.6° and a fall of only 9.6°. It is concluded that Atherinops, like Girella, certainly is not any more resistant to cooling than to warming. The importance of lethal cold as a possible limiting factor in the distribution of sensitive marine fishes, and of acclimatization as a factor in their dispersal, has now been amply demonstrated.[9] When all of the pertinent data are considered, it becomes evident that both Atherinops and Girella almost certainly cannot survive long at 0° even after very gradual cooling, the true range of thermal tolerance for Girella being smaller than that plotted by

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[7] $Q_{10} = (K_1/K_2)^{10/(t_1 - t_2)}$, where K_1 and K_2 are velocity constants at temperatures t_1 and t_2, respectively, of which t_1 is the higher.

[8] Surface temperatures reported for the northern portion of the reputed range of distribution of *Atherinops affinis* (United State Weather Bureau, 1938) average 11° to 13°, corresponding winter averages being about 1° lower. However, the species may be represented in different portions of its range by physiologically different races, the resistance of which to cold may be inherently different.

[9] For data on the reactions of Fundulus and Atherinops, as well as of Girella, to temperature gradients, which also indicate a pronounced sensitiveness of these fishes to cooling, see Doudoroff (1938).

Brett (1944), who used the author's data, but plotted only 12-hour tolerance limits. The susceptibility of *Fundulus parvipinnis* to chilling is less pronounced, but its ability to withstand 0° indefinitely after slow cooling is questionable, and this species is correspondingly resistant to heat.

There is evidence that not only the speed, but also the cause of death after chilling can vary with the intensity of the cold. The rapid death of Fundulus at very extreme low temperatures was quite different from the delayed death which followed more or less prolonged temporary recovery from the initial effects of cooling at less extreme temperatures. Very brief recovery never was observed at intermediate lethal temperatures. Only at the slowly lethal temperatures was death preceded in sea water by evident dehydration of tissues and delayed significantly by dilution of the medium.

The observation that recovery from primary chill-coma could be induced by warming and the administration of artificial respiration when warming alone was not sufficient suggests respiratory inhibition (Weigmann, 1936) as a cause of death at rapidly lethal low temperatures. When respiration is not resumed within a short time after cooling, asphyxiation or carbon dioxide narcosis may prevent subsequent recovery. However, at low temperatures hardy fishes can survive for hours in deoxygenated water (Sumner and Doudoroff, 1938). It is not improbable that asphyxiation was of importance as a cause of death only after warming (i.e., return to a normal temperature) and the consequent increase of the rate of respiratory metabolism.

At slowly lethal low temperatures increasing distress, indicative of cumulative injury, was evident long before the permanent cessation of regular respiratory movements, and the behavior of Fundulus did not suggest asphyxiation. It appears that at these temperatures some osmoregulative functions, such as the ingestion of sea water or the active excretion of salts by the gills, which compensate for the passive exosmotic loss of water through the external membranes (Smith, 1930; Keys, 1933), were inhibited, and in the normal medium dehydration and death followed as a result. The failure of these functions probably was itself a consequence of cold injury to tissues involved in osmoregulation (e.g., of the gills, the central nervous system or the integument) and was not, therefore, the primary cause of injury. The fact that mortality, although it was much delayed, was not entirely prevented when dehydration was avoided by dilution of the medium indicates that other, less rapidly lethal disturbances occurred also. Osmoregulative failure is only one of a number of possible causative factors which should be taken into consideration in further studies of the phenomena of chilling.

SUMMARY

The resistance of the hardy marine fish *Fundulus parvipinnis* and of the delicate *Atherinops affinis* to low and high temperatures is examined in relation to temperatures of previous acclimatization and to temperature conditions in the natural habitats of the fishes.

The relationships between the time (duration) of exposure to test temperatures and the average (median) limits of temperature tolerance indicate the value of prolonged tests in the study of resistance to cold and heat.

Marked susceptibility of marine fishes to chilling (i.e., injury at non-freezing

low temperatures) and the influence of acclimatization upon temperature tolerance are confirmed.

The death of Fundulus at slowly lethal low temperatures, but not at rapidly lethal temperatures, is preceded in the normal medium (sea water) by evident dehydration of the tissues and is delayed by dilution of the medium. Osmoregulative failure is indicated as one of the causes of slow death at low temperatures in sea water, but not of rapid death at more extreme low temperatures, which may be caused partly by respiratory disturbances.

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ORIGIN AND FUNCTION OF THE PROTOPLASMIC CONSTITUENTS IN PELOMYXA CAROLINENSIS

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Introduction

The protoplasmic constituents of several species of *Pelomyxa* have been described by Greeff (1874), Gould (1894), Wilson (1900) and Wilber (1942) and some suggestions have been made concerning their origin and their function but none of these suggestions are supported by experimental evidence. It, therefore, seems desirable to study the origin and function of these structures experimentally.

Relative Specific Gravity of the Protoplasmic Constituents

Open bottom hematocrit tubes were made of pyrex glass. The smaller opening was closed with a rubber band and the tubes were half filled with a heavy solution of gum-arabic in culture fluid. Then about 1 cc. of culture fluid, containing 5 or 10 pelomyxae, was carefully added and the solutions at the interface stirred with a glass rod and mixed so as to produce in this region a gradient of density and viscosity. The tubes were then put into an electric centrifuge and rotated, after which the rubber bands were quickly removed and the contents poured into partially frozen culture fluid, so as to prevent recovery of the pelomyxae. Some of these centrifuged, chilled pelomyxae were put into each of the following: one per cent osmic acid in culture fluid, ninety-five per cent alcohol, Champy's fluid, Regaud bichromate-formol mixture, and Bouin's fluid. These preparations were then studied under high and low magnifications. The results obtained are presented in Figure 1 and the following paragraphs.

Figure 1 shows that the pelomyxae during centrifugation were much elongated, that the refractive bodies (Wilber, 1942) aggregated at one end and the fat globules and contractile vacuoles at the other and that the food-vacuoles, the nuclei, the crystals, the beta granules, and the clear hyaloplasm respectively in fairly definite layers between; but it does not show which end is heavier.

Numerous specimens were consequently withdrawn from the tubes immediately after centrifugation, by means of an ice-cold capillary pipette of such small bore

[1] The writer wishes to express his sincere gratitude to Dr. S. O. Mast, who suggested the problem and under whose direction the work was done, for constant encouragement and invaluable aid in preparing the manuscript.

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that they could not turn end for end. The capillary was then immersed in nujol, to eliminate distortion caused by the curved side walls, and examined under the compound microscope. Invariably, the refractive bodies were at the centrifugal and the fat globules at the centripetal end. The refractive bodies, consequently, are the heaviest and the fat globules and contractile vacuoles the lightest constituents of *Pelomyxae carolinensis.* Mast and Doyle (1935) obtained similar results in observations on *Amoeba proteus.* However the substance in the nuclei did not stratify as in *Amoeba proteus,* indicating that either all the nuclear material has the

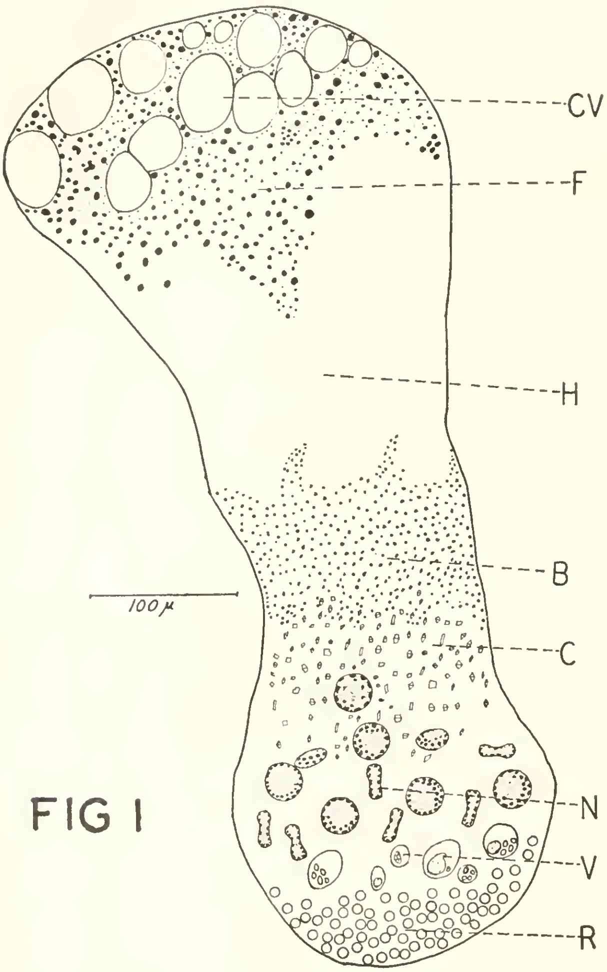

FIG I

FIGURE 1. Camera sketch of a centrifuged *Pelomyxa* showing stratification of the protoplasmic constituents. R, refractive bodies (at centrifugal end); V, food-vacuoles; N, nuclei; C, crystals; B, beta granules (mitochondria); H, layer containing only hyaloplasm; F, fat; Cv, contractile vacuoles.

same specific gravity or that the viscosity of it is so high that the granules do not stratify under the centrifugation used.

The crystals were free in the cytoplasm, and not in vacuoles as occurs very commonly in uncentrifuged specimens and in centrifuged amoebae (Mast and Doyle), and many of them were broken probably by striking one another during centrifugation. Apparently, at the centrifugation used the crystals were thrown out of the vacuoles and aggregated in a different layer.

Recovery of Centrifuged Pelomyxae

Pelomyxae were centrifuged as described above and put on slides and examined under high magnification while the temperature slowly increased to that of the room. As the temperature increased, the plasmasol (Wilber, 1942) began to flow toward the centripetal end in all but a few specimens in which very small, stubby pseudopods first formed at the centrifugal end but these were soon withdrawn and the plasmasol began to flow in the opposite direction. The refractive bodies were the first protoplasmic constituents to enter the flowing plasmasol. They were carried toward the centripetal end and became distributed in the lower layer of the plasmasol. After the refractive bodies were all in the stream the food-vacuoles were carried forward and came to lie above the refractive bodies; then the crystals and beta granules were carried forward. In this manner the protoplasmic constituents were redistributed through the cell. The pelomyxae remained in the monopodal form during and for some time after the redistribution. The entire process required about 2 minutes.

Function of Nuclei

Pelomyxae were mounted on a slide without a cover-slip, examined under the high power of the binocular dissecting microscope, and left undisturbed until they had attached and had extended one or more pseudopods. Then, with the aid of a fine glass needle, pseudopods which contained refractive bodies, crystals, beta granules, and contractile vacuoles, but only a few or no nuclei, were cut off and mounted on a slide under a cover-slip supported by a ring of vaseline, some with numerous chilomonads, others with no food. The slides were examined under the low and high power of the compound microscope to ascertain the effect of removing various numbers of nuclei, on locomotion, feeding, digestion, and longevity.

Locomotion: After the operation, most of the fragments remained quiet for 30 to 120 minutes. Then all except those with no nuclei became active and soon moved precisely like normal specimens in all respects, and sudden increase in the intensity of light caused them to stop moving, just as it does normal specimens. Moreover, the method of formation of pseudopods was the same as in normal pelomyxae except that those with less than 9 or 10 nuclei invariably assumed the monopodal form which is not characteristic of uninjured pelomyxae.

All the anucleate fragments were sluggish. Coarsely granular material was aggregated in the center leaving a peripheral layer with no cytoplasmic constituents except beta granules; but usually there was no clear hyaline layer, although some few did have a distinct hyaline cap. The process of locomotion was greatly modified. In fact, most of the fragments did not move at all unless stimulated strongly.

These results are like those obtained by Verworn (1909), Gruber (1912), Stolć (1910) and Willis (1916) in observations on *Amoeba.*

Feeding and Digestion: Numerous fragments obtained as described above were examined carefully. All nucleate fragments ingested food and the process was the same as in uninjured pelomyxae. Fragments with 1, 3, 6, and 10 nuclei respectively were stained with neutral red and the process of digestion carefully observed. No difference from the normal was evident. Refractive bodies were formed as in normal specimens. The increase in number observed in 36 hours after feeding is given in Table I. This table indicates that the number of refractive bodies formed varied inversely with the number of nuclei.

TABLE I

Table showing the relation between the number of nuclei and the increase in number of refractive bodies in nucleate fragments of *Pelomyxa carolinensis.*

Number of nuclei in each fragment	Number of refractive bodies		Increase in per cent	Average
	Before feeding	36 hours later		
1	10	16	60	63
1	3	5	66+	
3	9	13	44+	45
3	4	5	25	
3	6	10	66+	
6	12	17	41+	39
6	8	11	37+	
10	31	30	0	0

Ten anucleate fragments were studied. Seven of these did not ingest anything; three ingested one chilomonad each. One of these was observed during the process of ingestion. It formed a food-cup which was apparently normal. The ingested chilomonads died in the food-vacuoles but were not digested. One of the fragments was sketched 24 hours after it had ingested a chilomonad (Fig. 2F). The chilomonad was then dead but still intact and there was no evidence of digestion even after 72 hours. A partially digested chilomonad was in a food-vacuole in this fragment at the time of operation; digestion proceeded no further (Fig. 2F′).

These results indicate that anucleate fragments of *Pelomyxa* sometimes ingest food and kill it but that they do not digest it, or food which was ingested before the operation. The results agree in part with those of Stolć (1910) who maintains that anucleate amoebae ingest food normally for several days. They are not in accord with the views of Hofer (1890), Gruber (1912), Willis (1916), Lynch (1919), and Becker (1926) who contend that anucleate amoebae do not ingest food. Becker, however, maintains that in anucleate fragments of *Amoeba dubia* "Food once ingested can be digested by the amoebae after the fashion of normal amoebae."

Length of life: Numerous fragments with different numbers of nuclei but with the same number of beta granules, crystals, and refractive bodies (Wilber, 1942) were obtained as described above. Great care was taken to have the volume of the various fragments as nearly equal as possible. They were all passed through five

TABLE II

Table showing the relation between the number of nuclei and the length of life in fragments of *Pelomyxa carolinensis* in the absence of food.

Number of nuclei	Number of food-vacuoles	Number of refractive bodies	Average	Length of life in days	Average
1	0	10	6.2	8	6.2
1	1	3		4	
1	0	5		6	
1	1	7		7	
2	2	3	5.6	8	6.3
2	0	8		6	
2	0	6		5	
3	0	4	7.0	5	7.5
3	2	10		10	
4	1	12	6.0	6	8.5
4	2	3		10	
4	2	3		10	
4	0	6		8	
5	1	4	8.0	8	8.3
5	9	12		7	
5	1	8		10	
8	2	12	11.0	19	19.0
8	1	8		23	
8	3	13		15	
10	2	14	12.3	17	18.6
10	1	12		14	
10	3	11		25	
12	1	15	16.0	21	20.0
12	3	17		19	
20	3	20	18.6	23	24.0
20	3	19		19	
20	3	17		30	
25	2	21	18.5	26	23.5
25	1	16		21	
40	2	16	23.0	21	24.0
40	2	30		27	
50	4	18	22.6	27	28.6
50	4	10		29	
50	3	40		30	

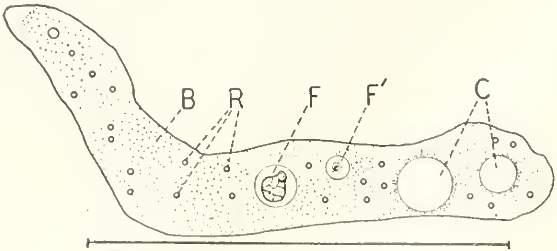

FIG 2

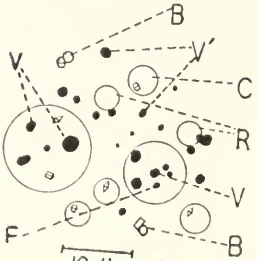

FIG 5

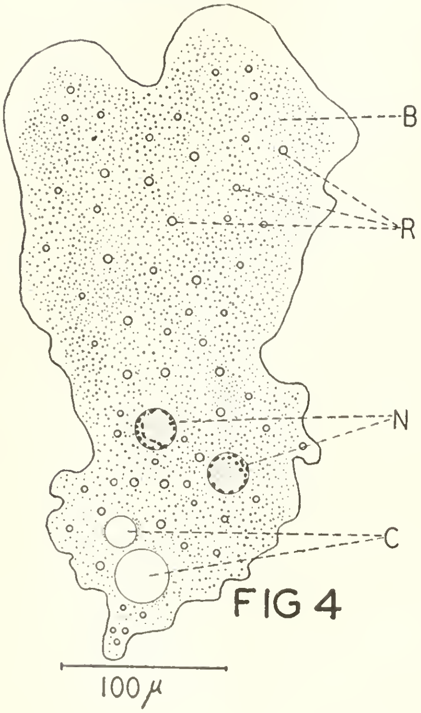

FIG 4

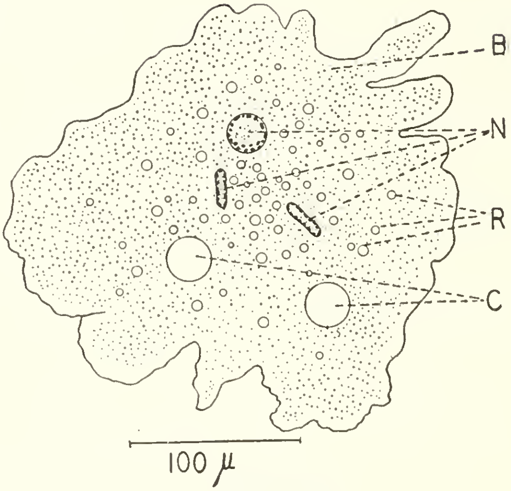

FIG 3

Figures 2–5.

Figure 2. Camera sketch of anucleate fragment of *Pelomyxa carolinensis*. C, contractile vacuoles; F, food-vacuole containing a chilomonad which was ingested after all the nuclei had been removed; F¹, food-vacuole present before the operation; R, refractive bodies; B, beta granules.

Figure 3. Camera sketch of a fragment of a pelomyxa one minute after it had been cut off. B, beta granules; C, contractile vacuoles; N, nuclei; R, refractive bodies. The fragment had no contractile vacuoles when it was cut off. Note that one minute later, two contractile vacuoles had formed, but that there was no aggregation of beta granules around them. They contracted about one hour after the sketch was made but there was no indication of aggregation even then.

Figure 4. Camera sketch of a fragment of a pelomyxa one hour after it had been cut off. N, nuclei; C, contractile vacuoles; R, refractive bodies; B, beta granules. The fragment had no contractile vacuoles until shortly before the sketch was made. Note that a few beta granules

separate 10 cc. portions of sterile culture fluid, then each was put into 5 cc. of this fluid in a small glass dish and observations made on the relation between the number of nuclei and the length of life.

When the fragments failed to respond to stimulation with a fine glass rod, they were tentatively considered dead. When they showed evidence of disintegration, they were pronounced dead. The occurrence of these two phenomena never differed by more than four hours. The results obtained are given in Table II. This table shows that the fragments with one to 5 nuclei lived roughly the same length of time, but that those with more than 5 nuclei lived much longer.

It may be contended that this is due to differences in the number of refractive bodies and food-vacuoles in the fragments or to differences in their size. As stated above, all the fragments were nearly the same in size. The difference in the length of life observed could therefore not have been due to difference in size. The table shows that while in the fragments with 5 or fewer nuclei the length of life usually varied directly with the number of refractive bodies and food-vacuoles, in those with more than 5 nuclei this did not obtain. This seems to show that while the length of life of fragments of *Pelomyxa* is, at least under some conditions, dependent upon the number of refractive bodies and food-vacuoles present, it is also dependent upon the number of nuclei present.

The evidence presented, then, seems to show that the presence of at least one nucleus is necessary for locomotion and digestion but not for ingestion of food, and that the length of life of a fragment, without food, varies directly with the number of nuclei and the number of food-vacuoles and refractive bodies present.

Formation of the Contractile Vacuoles and the Relation Between Them and the Beta Granules

Numerous pelomyxae were examined under the high power of a binocular dissecting microscope. The animals were left undisturbed on a slide without a cover-slip until they had attached and had extended one or more pseudopods. With the aid of a fine glass rod, pseudopods which contained one or more nuclei, refractive bodies, crystals, and beta granules, but *no contractile vacuoles,* were cut off and mounted on a slide under a cover-slip supported by a ring of vaseline, and examined under the oil immersion objective of a compound microscope.

The following was observed: New contractile vacuoles formed in all the fragments. The time for the formation of new contractile vacuoles varied from about 60 seconds to more than one hour after the operation (Fig. 3). The vacuole was first visible as a small fluid-filled space in the hyaloplasm, no larger than a beta granule. This grew rapidly in size until it was about 5 micra in diameter and then enlarged more slowly until it contracted. At no time was there an aggregation

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have aggregated around them. Ten minutes after this sketch was made, the two vacuoles coalesced and another one formed between the two nuclei.

Figure 5. Camera sketch of portion of cytoplasm in a starved pelomyxa twenty-four hours after it had ingested a colpidium. F, food-vacuoles; V, vacuole-refractive bodies in the food-vacuoles; V′, vacuole-refractive bodies in the cytoplasm; B, bleb with a crystal attached to it; C, crystal in a vacuole; R, vacuole-refractive bodies in contact with blebs. Note that some of the food-vacuoles contain crystals and vacuole-refractive bodies. Some of the vacuole-refractive bodies are free in the cytoplasm. Several are in contact with blebs formed from the crystals. Note also two crystals with blebs and crystals in vacuoles.

of beta granules in the region where the vacuole formed but after it had discharged a few times, beta granules aggregated around it (Fig. 4).

The time required for the beta granules to aggregate around the contractile vacuole varied greatly. In some of the fragments studied, there was no sign of aggregation for several days after the vacuole had formed and begun to function; in others, aggregation began very soon after it had formed. The presence or absence of nuclei in the fragments had no apparent effect on the time of formation of the contractile vacuole and the variation in time was as great in anucleate as in nucleate fragments, and the nucleus had no effect on the time required for the aggregation of beta granules around the vacuole.

In uninjured pelomyxae, the cytoplasm immediately around the contractile vacuole is more viscous than that which is elsewhere in the plasmasol (Wilber, 1942). Young contractile vacuoles, however, i.e., vacuoles which had recently arisen *de novo,* were seen often to come into intimate contact with nuclei, food-vacuoles, and other structures in the cytoplasm, and the beta granules in the cytoplasm adjoining them had as marked Brownian movement as those elsewhere in the plasmasol. It was not until after the first contraction that evidence of higher viscosity was observed in the cytoplasm adjoining the surface of the newly formed vacuoles. These facts indicate that the cytoplasm adjoining the surface of the contractile vacuole does not become viscous until some time after the vacuole has arisen. After a viscous layer has formed around a contractile vacuole, the latter always expands in this viscous substance after each contraction. New vacuoles, however, arise elsewhere in the cytoplasm.

It thus appears that in *Pelomyxa* the beta granules are not directly related to the formation of contractile vacuoles and that their aggregation and the gelation of the cytoplasm around the contractile vacuoles are in no way necessary for the action of the vacuoles.

These results are in accord with the contention of Mast (1938), in reference to *Amoeba proteus* but not with those of Howland and Pollack (1927) in reference to *Amoeba verrucosa.* Mast says: "The differentiation of a layer of substance on the surface of the contractile vacuole is probably due to the action of the fluid in the vacuole on the adjoining cytoplasm," and "neither the beta granules nor the layer of viscous substance is involved in the function of the contractile vacuole." Howland and Pollack (1927) maintain that the gelated region around the contractile vacuole can "be considered as supplying the initial impulse for systole. . . ." This is not true in *Pelomyxa carolinensis* for in it the gelated layer does not form until after the first contraction. It is, therefore, not necessarily involved in the contraction of the vacuole and it obviously does not necessarily function in the accumulation of fluid in the vacuole.

It can, therefore, be concluded that neither the beta granules nor the viscous cytoplasm around the contractile vacuoles is of primary importance in the formation or the function of the contractile vacuoles.

Function of the Beta Granules

Wilber (1942) demonstrated that the beta granules in *Pelomyxa carolinensis* stain like mitochondria and he concludes: "These granules, like the beta granules of *Amoeba proteus* (Mast and Doyle, 1935), are consequently similar in composition to the mitochondria in metazoan cells." Metcalf (1910), referring to *Amoeba* *proteus,* holds that they are involved in the excretion of substance by the contractile vacuole and he consequently calls them "excretory granules."

Mast and Doyle (1935) attempted to ascertain their function in *Amoeba proteus* by centrifuging and then cutting out different proportions and noting the effect and by observing their movements in normal specimens. They concluded that "the beta granules function in accumulation of fluid eliminated by the contractile vacuole." They are the only investigators who approached the problem experimentally.

In the present work, the experiments and observations of Mast and Doyle were repeated on *Pelomyxa carolinensis.* Some specimens were centrifuged and cut at different levels so as to remove various constituents and different proportions of the beta granules (Fig. 1). Others not centrifuged were cut, to obtain fragments of approximately the same size as the centrifuged fragments, but with normal proportions of all the protoplasmic constituents. Both kinds of fragments were mounted in culture fluid, without food, on slides under cover-slips supported by rings of vaseline and studied until all the fragments had died. This was repeated five times. The results obtained in a typical experiment are presented in Table III.

TABLE III

Table showing the effect of removing different protoplasmic constituents on the length of life of pelomyxae. F, fat; Cv, contractile vacuoles; H, hyaloplasm; B, beta granules.

Constituents removed	Number of specimens	Average length of life in days	
		Constituents removed	No constituents removed
F + Cv	10	18	21
F + Cv + $\frac{1}{2}$H	9	7	22
F + Cv + H	10	1	19
F + Cv + H + $\frac{1}{2}$B	8	1	20
F + Cv + H + B	8	$\frac{1}{4}$	15

Table III shows the following: The uncentrifuged fragments lived, on an average, from 15 to 22 days. The centrifuged fragments produced by cutting so as to remove the fat and contractile vacuoles lived nearly as long as the uncentrifuged fragments. Those produced by cutting so as to remove the fat, contractile vacuoles, and about half of the hyaloplasmic layer lived about one-third as long. Those produced by cutting so as to remove practically all the hyaloplasmic layer lived only about one-twentieth as long, but those produced by cutting so as to remove approximately half of and all the beta granules respectively showed no marked further decrease in length of life.

It is evident from these results that the removal of the fat and the contractile vacuoles causes but little, if any, decrease in length of life, that removal of a large proportion of the hyaloplasm, in addition to the fat and the contractile vacuoles, causes a pronounced decrease in the length of life, and that the removal of the beta granules in addition to the hyaloplasm, fat, and contractile vacuoles causes little, if any, further decrease in length of life. These facts indicate that length of life in fragments of *Pelomyxa* is primarily dependent on the amount of hyaloplasm in them, and only secondarily, if at all, on the number of beta granules.

The results are in harmony with the contention of Holter (1936) and Holter and Kopac (1937) that enzymes in amoebae and in marine ova are localized in the hyaloplasm and not in any formed bodies, and also with the views of Just (1939) in regard to the great importance of the ground cytoplasm in cellular function.

The facts presented, therefore, seem to indicate that beta granules (mitochondria) do not play an active part in cell functions and that their observed movements are merely a visible sign of submicroscopic changes in the hyaloplasm.

Formation of Refractive Bodies

About 100 pelomyxae were centrifuged and cut so as to remove most of the fat and all the refractive bodies (the fat was removed so that it could not serve as a source of food). Half of them were then put into each of two dishes containing sterile culture fluid and numerous colpidia added to the fluid in one of the dishes. In about 50 minutes all the specimens had recovered from the "shock" of the operations, as indicated by the facts that they moved normally and responded to increase in intensity of light by cessation of movement.

Two of the 50 pelomyxae without food died 20 hours after the operation; four of them lived until the 7th day; none divided. The average length of life was five days. No vacuole-refractive bodies (Wilber, 1942) or refractive bodies formed in any of them. Three of the 50 pelomyxae with food did not feed and died four hours after the operation. Each of the others ingested one or more colpidia. Twenty-four hours after feeding, there were numerous vacuole-refractive bodies in the food-vacuoles and some in the cytoplasm of every one of these specimens, and some of the food-vacuoles in a few of them contained crystals. These results show that food is necessary for the formation of vacuole-refractive bodies and of refractive bodies, and that the crystals are formed in the food-vacuoles. They also show that removal of the refractive bodies has no apparent injurious effects on pelomyxae. This is in accord with the views of Mast and Doyle (1935) who contend "that the removal of all the refractive bodies does not seriously interfere with the vital processes in *Amoeba*," but is not in accord with the views of Singh (1938) who maintains that specimens of *Amoeba proteus* with the refractive bodies removed appear "as if they were encysted until these bodies are formed," and that "as soon as these bodies are formed, the amoebae regain their normal shape and activity."

Ten pelomyxae were kept in sterile culture fluid for 15 days; then colpidia were added and each pelomyxa examined under low and high magnifications at intervals of one hour. The results obtained in observations on all these pelomyxae are in accord with the following detailed description concerning one which had ingested two colpidia: One hour after ingestion, the two colpidia were dead, but there was no evidence of digestion. Two hours after ingestion, both colpidia stained deeply red with neutral red, but the fluid in the food-vacuoles did not stain; the vacuolar membrane was very close to the pellicle of the colpidia. Three hours after ingestion, the colpidia stained deeply red in neutral red, and the fluid in the vacuoles, orange; the vacuoles had enlarged, probably as a result of fluid passing in from the cytoplasm. Four hours after ingestion, both food-vacuoles had divided; fragments of the colpidia in the four resulting vacuoles stained deeply red; two of the vacuoles contained vacuole-refractive bodies which stained intensely red in neutral red (one vacuole had three; the other, one). Six hours after ingestion, there were six food-vacuoles present; all contained vacuole-refractive bodies; two contained hyaline nonstaining spherical bodies about 4 micra in diameter and of unknown composition. Twelve hours after ingestion, there were numerous vacuole-refractive bodies in each of the six vacuoles and several crystals in four of them; the other two contained no crystals; the vacuole-refractive bodies varied from 1 micron to about 6 micra in diameter; several were free in the cytoplasm; the manner in which they entered the cytoplasm was not ascertained. Twenty-four hours after ingestion, there were eight food-vacuoles present; five contained vacuole-refractive bodies and crystals, three, only vacuole-refractive bodies; there were numerous vacuole-refractive bodies and several crystals in the cytoplasm; some of the crystals had blebs attached (Fig. 5).

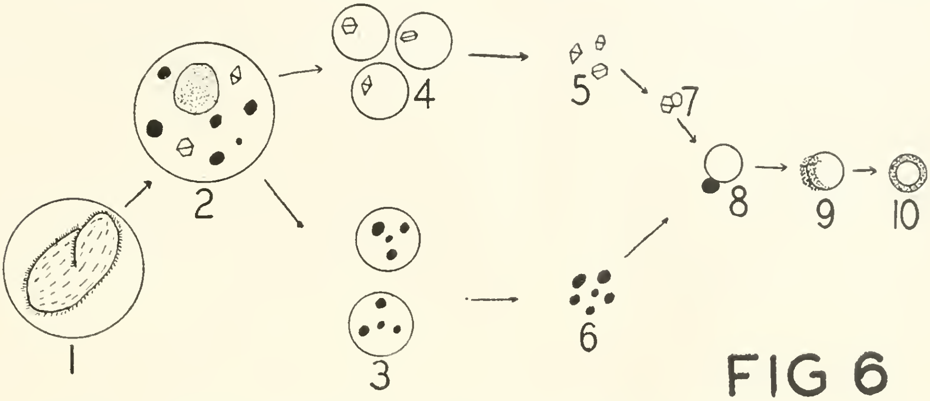

FIG 6

Figure 6. Diagram illustrating the formation of the refractive bodies from digested food. 1, food-vacuoles containing an ingested organism, usually a colpidium or a chilomonad; 2, food-vacuole containing a partially digested organism and crystals and vacuole-refractive bodies formed from the food during the process of digestion; 3 and 4, vacuoles formed by the division of 2; 5 and 6, crystals and vacuole-refractive bodies free in the cytoplasm; the manner in which they leave the vacuoles is not known; 7, crystal with bleb attached to it (the crystal disappears and the bleb greatly enlarges); 8, enlarged bleb in contact with a vacuole-refractive body; 9, vacuole-refractive body "flowing over" the bleb; 10, refractive body formed by the union of a bleb and a vacuole-refractive body. Stippling in 9 and 10 represents material of vacuole-refractive body.

One of the crystals with a bleb attached was observed closely under oil immersion for several hours. The bleb became larger and simultaneously the crystal smaller until it was no longer visible. Soon another crystal came in contact with the bleb, and in about 40 minutes it too disappeared and the bleb became still larger. The crystals were obviously used in the growth of the bleb. Fifteen minutes later, the bleb came in contact with three vacuole-refractive bodies which were free in the cytoplasm. The substance in these three bodies "flowed over" the bleb and covered it with a layer, resulting in the formation of a body which was identical with other refractive bodies. The time required was 65 minutes. The beta granules took no visible part in the formation of the refractive body. Similar results were obtained in observations on crystals with blebs attached and vacuole-refractive bodies in normal pelomyxae.

The present observations clearly indicate that both the crystals and the vacuole-refractive bodies originate from the food in the food-vacuoles, that they leave the vacuoles and are found free in the cytoplasm (the manner in which this takes place is not known), and that the crystals form nonstaining spherical bodies (blebs) which become covered with a layer of substance from the vacuole-refractive bodies and thus form refractive bodies (Fig. 6).

The conclusions concerning the formation of the refractive bodies from the crystals and the vacuole-refractive bodies are in accord with the contention of Mast and Doyle (1935) who maintain that "The refractive bodies . . . originate and develop in the cytoplasm probably from substances obtained from the vacuole-refractive bodies and the crystals."

Mast and Doyle (1935) maintain that the beta granules function "in transferring digested substances from the food-vacuoles and the crystal vacuoles to the refractive bodies." There is no indication of such a function in *Pelomyxa.*

FUNCTION OF THE REFRACTIVE BODIES

Fifty pelomyxae were passed through five different portions of sterile culture fluid and then put into a stender dish containing 25 cc. of this fluid but no food. Each day a few were stained with neutral red, Sudan black, and Janus green B respectively, and these and a few unstained specimens examined for refractive bodies, fat, beta granules, and crystals. The results are presented in Table IV.

TABLE IV

Showing the decrease in number of refractive bodies in the absence of food.

Date	Number of refractive bodies in each of 9 pelomyxae									Average
	a	b	c	d	e	f	g	h	i	
Sept. 25	50	20	40	50	62	31	23	41	40	39.7
Sept. 30	42	18	32	—	50	27	17	34	30	31.2
Oct. 1	30	10	30	33	50	26	13	30	30	28.0
Oct. 2	23	10	27	27	—	26	13	30	23	22.3
Oct. 3	20	8	27	27	43	—	11	—	23	22.7
Oct. 4	17	8	23	24	43	23	11	25	20	21.5
Oct. 5	14	8	20	20	41	21	11	25	19	19.8
Oct. 10	14	8	17	20	37	15	7	21	10	16.4

This table shows that there is in *Pelomyxa carolinensis* a steady decrease in the number of refractive bodies during starvation. It was observed that some of the refractive bodies disintegrated and that during disintegration the outer layer gradually disappeared, leaving a homogeneous mass which did not stain neutral red. These results indicate that the refractive bodies function as reserve food. This conclusion is in accord with that of Mast and Doyle (1935) and Singh (1938) in regard to the function of the refractive bodies in *Amoeba proteus.*

SUMMARY

1. Centrifugation causes the protoplasmic constituents of *Pelomyxa carolinensis* to stratify into definite layers which, in order of decrease in weight, are as follows:

refractive bodies, food-vacuoles, nuclei, crystals, beta granules, hyaloplasm, contractile vacuoles, and fat.

2. The presence of at least one nucleus is necessary for normal locomotion and digestion, but not for ingestion of food in *Pelomyxa*. The length of life of *Pelomyxa* during starvation varies directly with the number of nuclei present.

3. The contractile vacuoles arise *de novo* in the hyaline cytoplasm. Their formation and functioning is not dependent on the presence of nuclei or beta granules.

4. The beta granules (mitochondria) do not play an active role in cell functions but, probably, merely give visible evidence of submicroscopic changes taking place in the hyaloplasm.

5. The vacuole-refractive bodies and the crystals are formed from the food in the food-vacuoles of *Pelomyxa* and are used in the formation of the refractive bodies.

6. The refractive bodies function as food reserve in the cell. Their complete removal does not impair any of the cellular activities.

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STAGES IN THE DEVELOPMENT OF THE PICKED OR SPINY DOGFISH, SQUALUS ACANTHIAS LINN.

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Introduction

In a previous paper, von Bonde (1945), I described various features of the external development of the oviparous Banded Dogfish, *Haploblepharus edwardsii*. The present species, which attains a length of 915 mm., belongs to the group whose development is classified as ovoviviparous. This species (Plate I, Fig. 1) is very common in the Cape and Natal waters of South Africa and its distribution ranges along the Atlantic coasts of Africa, Europe, and North America and to the Mediterranean.

An egg-case containing three developing embryos (Plate II) was laid by a female in one of the tanks of the Sea Point Aquarium.

Female Reproductive Organs

The only secondary external sexual characters in the female are the pair of cloacal papillae situated one on either side of the median line immediately posterior to the cloacal aperture. The female contains a paired ovary situated in the usual position towards the anterior end of the coelom. These ovaries (Plate I, Fig. 2, r.ov., l.ov.) change in shape and size according to whether the female is gravid or not, although the cycle of the ovarian growth of the ova is such that ripe ova are again in evidence immediately after the young are born. The paired oviducts are not so highly differentiated in structure and function in their anterior half as those of *Haploblepharus edwardsii,* since the ovum or ova is usually enclosed in a very thin transparent chitinous capsule which is not as highly specialized as the egg capsules or mermaid's purses of the oviparous species. The posterior half of each oviduct, as will appear later, is much more highly developed and specialized in conformity with the ovoviviparity of this species. The oviducts meet in the middle line at the anterior end of the coelom and open into the body cavity by a single orifice. By making a transverse incision immediately posterior to the edge of the pectoral girdle and carrying this incision laterally and then anteriorly and lifting the ventral muscular wall, the confluence of the oviducts is exposed. This common opening of the oviducts is attached by a mesentery in the middle line to the surface of the ventral wall of the coelom (Plate I, Fig. 4, f.t.o. and m.). This common opening is wide and funnel-shaped and lies right over the antero-ventral part of the ovaries so that immediately after a ripe ovum escapes from the ovary it is taken up by the lips of the funnel and passed backward along the fallopian tube (Plate I, Fig. 4, f.t.).

Each oviduct may be divided into five distinct portions as follows:

(1) The fallopian tube (Plate I, Figs. 2 and 4, f.t.) is extremely narrow, thin-walled and comparatively short. This portion is followed by (2) the albumen gland (a.g.) which in this species is very small, thick-walled and muscular, the internal surface being richly supplied with glands secreting the albumen which covers the ovum. The albumen gland is followed by (3) the nidamental gland (n.g.) which is a long thin tube of about the same diameter as the fallopian tube. Here the transparent chitinous egg capsule is secreted. The nidamental gland opens posteriorly into (4) a very wide saccular uterus (u.) in which the development of the embryo takes place. The uterus in turn narrows into (5) a short vagina (v.) which opens into the cloaca. In immature females the vaginal orifices are separated from the cloaca by a membrane or hymen which during copulation is pierced by means of the special claws of the claspers (see below). It is interesting to note that in those Elasmobranchs in which the shape of the claspers of the male is flat, the vaginal orifices are slit-shaped, while in those forms in which the claspers are provided with hooks, the lining of the vagina is thickened (Daniel, 1934).

The uterus, which was described in detail by Widakowich (1907a), is more or less typical of those species displaying viviparity or ovoviviparity. In *S. acanthias* each uterus is a greatly enlarged sac with well defined boundaries. The mucous membrane of each uterus varies greatly in conformity with the development of the ovaries. Thus, when the ovaries are large and contain ripe ova ready for fertilization, the mucous membrane of the uterus is more or less smooth. In gravid females, on the other hand, this mucous membrane is thrown into longitudinal rows of flaps known as papillae or villi, each of which is a leaf-like structure with very thin walls (Plate III, Fig. 10, p.). The border of each papilla is thickened by a blood vessel. The blood supply to the uterus is exceedingly profuse (Daniel, op. cit.). The arterial supply consists in part of the anterior and in part of the posterior oviducal arteries which break up into branches to the rows of papillae. Each terminal artery runs along the free borders of the papillae to supply each single papilla with blood. The blood from each papilla is drained by central veins into a main uterine vein. The two systems are connected by a capillary net around the surface of the papilla.

Ercolani (1879) described the viviparous Selachians according to the various aspects of the relationships between the developing eggs and the inner wall of the uterus, and, according to the degree of these relationships, he differentiated four classes:

1. Naked contact between the homogeneously smooth mucous membrane of the uterus and the surface of the egg (*Plagiostomi acotyledonale*). *Squatina angelus* and *Heptanchus cinereus* are cited by him as examples.

2. Complicated contact between both surfaces, the complication being due to the presence of numerous folds in the secreting mucous membrane of the mother which causes an appreciable increase of the inner surface (*Plagiostomi acotyledonale*). *Squalus acanthias* and *Scymnus lichia* are cited as examples.

3. The complicated contact is differentiated by a more or less significant new structure of papillae on the folds of the secreting mucous membrane of the uterus by means of which its inner surface and activity are extraordinarily enlarged (*Alcuni selachii*). *Torpedo* and *Pteroplatea micrura* are cited as examples. (Note: According to the development of the papillae in the uterus it appears as though *S. acanthias* should be placed in this group rather than in Group 2.)

4. The relationship is no longer due to contact but to an intimate union between a part of the absorbing upper surface of the egg with a part of the secreting surface of the uterus. Both surfaces form folds which interlace and at the same time fuse with each other. Thus a type of rudimentary placenta is formed which is comparable with the mammalian placenta (*Plagiostomi cotyledonale*). *Carcharias glaucus* and *Mustelus laevis* are cited as examples.

In *S. acanthias* the papillae of the mucous membrane arise from a cutaneous development of the convoluted marginal arteries. The papillae are twisted around their longitudinal axes. The changing form of the papillae is due partly to a purely mechanical and partly to a reflex action. There appears to be no relationship between the gestation period and the length of the papillae. The uterus is supplied with blood from two capillary systems, viz., (a) the organotropic and (b) the embryonotropic. The latter forms a capillary network which covers the papillae and also the remaining part of the inner wall of the uterus. The capillary network receives its blood from the marginal arteries and releases it into the basal veins. These do not possess any separate musculature but are embedded in the muscular fasciculi of the uterus which open towards the papillae.

Widakowich (1907b) described the closing device ("Verschlussvorrichtung") situated between the portion I have designated as the nidamental gland and the dome of the uterus (Plate I, Fig. 2A, 2B, c.d.). The gravid uterus (Plate I, Fig. 2, u.) has a cylindrical shape with conical ends pointing anteriorly and posteriorly. The uterine arteries are strong walled (Plate I, Fig. 2A, a.) and run laterally along the nidamental glands. The uterine vein (ve.) runs in close proximity to the artery. The closing device is necessary since the uteri, as soon as the embryos reach a definite age and their enveloping membranes rupture, are filled with a large quantity of nutrient yolk, which, but for this closing device, would be forced along the whole length of the oviducts during any contraction of the uterus, or external pressure from the distension of the alimentary channel, or the movements of the embryos in the uteri should the yolk sac be ruptured. During the time of rut and fertilization there is a strong hyperaemia of the whole genital tract; hence the genital tract is well supplied with blood vessels. The function of the papillae above referred to will be dealt with later.

Male Reproductive Organs

The morphology of the male reproductive system follows the general plan of all Elasmobranchs. The testes are paired and occupy the anterior half of the

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Plate I

Figure 1. Left lateral view of *Squalus acanthias*.

Figure 2. Dissection of a gravid female showing the reproductive organs.

Figure 2A. Closing device (c.d.) in oviduct (after Widakowich).

Figure 2B. Internal surface of the dome at the anterior end of the uterus showing the closing device. The dome has been invaginated to bring the closing device into prominence, hence it appears nipple-shaped.

Figure 3. Egg capsule containing single ovum in early stages of development.

Figure 4. Portion of the anterior ends of fallopian tubes (f.t.) showing their confluence and coelomic orifice (f.t.o.).

Figure 5. Clasper of male.

Figure 6. Claw (cl.) and spur (sp.) of clasper greatly enlarged.

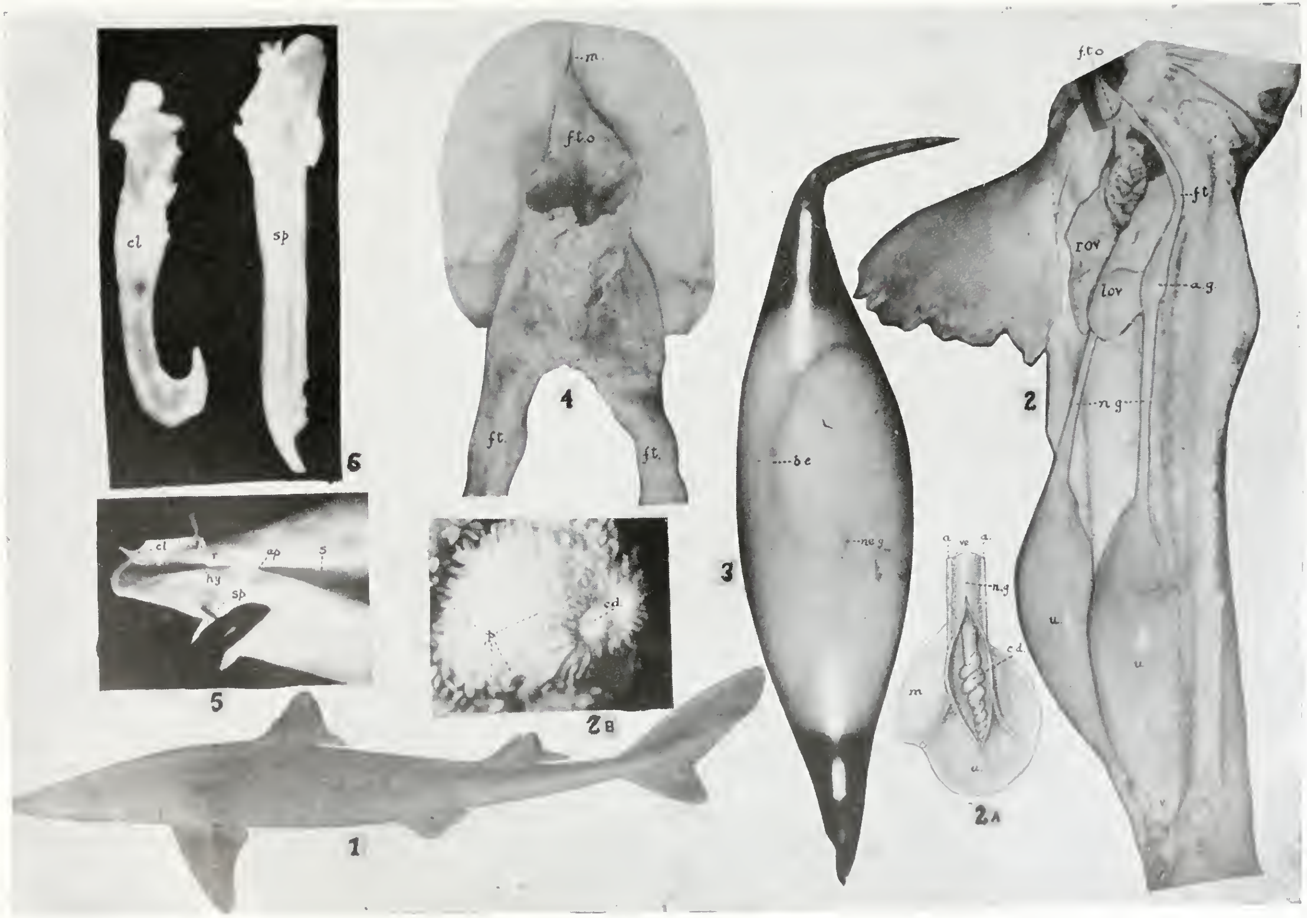
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Plate I

coelom. From the anterior end of each testis the vasa efferentia lead to the epididymus which is much coiled. The epididymus eventually widens out into the vas deferens which expands and joins its fellow in the middle line posteriorly, opening into the cloaca by a single pore.

The external secondary sexual characters of this species present certain distinctive features. As in all Elasmobranchs the basal element (basipterygium) of each pelvic fin is prolonged as a stout, backwardly-directed rod, sharply demarcated from the remainder of the fin, forming an intromittent organ, called the clasper or myxopterygium. A transverse section of the proximal half of the clasper shows it to be almost completely round, the internal cartilaginous skeleton forming a tube filled with a thick muscular substance through the center of which passes a duct. In the distal end the skeleton only occupies the ventral part of the clasper, being continued right to the tip. The latero-dorsal wings of this part of the clasper are formed of thickened skin, the outer wing being folded over the curved inner wing to form a canal which is continuous with the above-mentioned duct in the proximal half. This duct opens through the hypopyle into an open channel which is continued to the tip of the clasper (see Plate I, Fig. 5, hy.). On the outer surface near the hypopyle, a strong spiny spur (sp.) arises. On the inner ventral surface a claw (cl.), which extends to the tip of the clasper, is situated. This claw is much curved and, like the spur, is movable. Its function is probably to rupture the hymen. By pressing the distal end of the clasper, the spur is erected, and forms an important organ of attachment, penetrating and even lacerating the tissues of the female (Leigh-Sharpe, 1920). The spur in its retracted position lies alongside the claw in the same groove. The rhipidion (r.) in the normal intromittent position of the clasper covers the dorsal part of the groove and also the claw and the spur. The siphon (s.) is comparatively small and is situated immediately beneath the skin of the pelvic fin. The apopyle (ap.) lies some distance from the cloaca and relatively near to the hypopyle.

Development

It was not possible to study the complete development of the present species along the lines described in my previous paper (op. cit.) and it is, therefore, necessary to refer to the description of embryos of various lengths taken from gravid females. The gravid uteri shown in Plate I, Fig. 2, u. each contained a single egg capsule with a single ovum (Fig 3.), one being infertile.

A brief description of the primary phases in Selachian development is as follows: At the time of fertilization the ovum is usually in the shape of a large spherical mass, similar to the yolk of an avian ovum, but its shape may become roughly cylindrical as in the present case, the shape being determined by that of the enclosing egg capsule. The greater part of the egg is formed of peculiar oval spherules of food-yolk held together by a protoplasmic network (Balfour, 1881). The yolk is completely covered by the albumen which is thick and glairy and transparent except at the polar extremities. The protoplasm is concentrated in a small lens-shaped area, the germinal disc or "orange spot," and is situated much nearer to the broader end of the egg. Yolk spherules are also present in this disc, but are much smaller and of a different character. Segmentation is meroblastic or discoidal as in all telolecithal eggs, being confined to the germinal disc.

The blastoderm is thus formed and at the close of segmentation the blastoderm forms a lens-shaped disc thicker at one end than the other, the thicker end being known as the embryonic end where the future embryo develops. The three germinal layers become established and shortly after this the rudimentary embryo consists of an oblong plate which extends inwards from the periphery of the blastoderm. This plate is the medullary plate and along its axial line a shallow groove, the neural groove, is formed. The embryo now progressively increases in length and a certain number of mesoblastic somites become formed. The increase in length of the body takes place normally by growth in the region between the last mesoblastic somites and the end of the tail. The anterior part of the body is not completely folded off from the blastoderm and the neural groove becomes converted into the neural canal. As soon as the embryo has become folded off from the yolk both in front and behind, the separate parts of it are easily recognizable. The embryo is attached to the yolk by a distinct stalk, which gradually narrows and elongates and is known as the umbilical cord. The blastoderm continues to grow in all directions and by circumcrescence it provides a protective vascular covering of the yolk mass, but these extra embryonic structures will eventually be resorbed. The protective covering is developed from the ectoderm, whilst the vascular covering arises from the mesoderm. Before completely covering the yolk mass, however, at a certain stage a yolk blastopore, which is that portion of the surface of the yolk mass not yet covered by the blastoderm, is formed. The yolk blastopore is related to gastrulation since circumcrescence is an important factor in gastrulation and it assists in laying down the definitive endoderm. On the ventral surface of the head of the embryo the stomodaeum is formed, which eventually gives rise to the mouth, while some little distance from the posterior end of the embryo the proctodaeum will eventually give rise to the anus. At a somewhat later stage the gill clefts are formed, being at first six pairs in number, but soon the anterior pair becomes separated from the rest to give rise to spiracles. The first rudiment of the heart becomes visible during the 38 somite stage and has a cavity situated between the mesoderm and the endoderm. All the visceral clefts, including the spiracles, show long filiform external gills which project out for some distance from the sides of the head. These external gills lie against the front wall of the spiracle and the front walls of the visceral clefts. It appears as though they are specially developed to facilitate respiration during the earlier stages of development, either intra-uterine or intracapsular. They disappear some time before the close of the embryonic stage. The young Selachian has all the external characters of the adult when hatched, and can immediately swim about and fend for itself, but before hatching, the yolk has been completely absorbed by the developing embryo and the yolk sac and umbilical cord decrease in size until eventually they are only shown in the form of an umbilicus which persists for about 14 days after birth. The vitelline circulation becomes developed at a stage before the closure of the yolk blastopore. During the circumcrescence of the blastoderm its edge has remained thickened and this feature persists until the whole of the yolk is covered. In this thickened edge a circular vein arises which brings the blood back from the yolk sac to the embryo. The first stage in the development of the vitelline circulation is the formation of a single artery which emerges from the umbilical cord and proceeds cephalad along the blastoderm under cover of the head of the embryo. This arterial trunk divides to form two arcuate branches that turn laterad and then posterad towards the posterior margin of the blastoderm. These arterial branches eventually meet posteriorly and give off numerous small secondary branches, some of which connect with small veins emptying into a venous ring close to the margin of the blastoderm. The main trunk of the vitelline vein drains the venous ring and runs straight to the umbilical cord (Smith, 1942). When the yolk blastopore has completely closed, the venous ring disappears and the area formerly occupied by the yolk blastopore is traversed by a continuation of the main trunk of the vitelline vein. This trunk receives numerous small veins usually joining it at right angles. Since the venous trunks are only developed behind the embryo, it is only the posterior part of the arterial ring which gives off branches. At a still later stage the arterial ring embraces the whole yolk, but later vanishes in its turn, as did the venous ring before it. There is then present a single arterial trunk and a single venous trunk. The arterial trunk is a branch of the dorsal aorta, and the venous trunk enters the heart together with the splanchnic vein. The venous trunk enters the body on the right side and the arterial leaves on the left of the umbilical cord.

The yolk sac persists during the whole of the embryonic life and in the majority of Elasmobranch embryos there arises within the body walls an outgrowth from the umbilical canal into which a large amount of the yolk passes. This outgrowth forms an internal yolk sac. In viviparous species a remarkable peculiarity arises in that the vascular surface of the yolk sac becomes raised into a number of folds which fit into corresponding depressions in the vascular walls of the uterus. The yolk sac becomes in this way firmly attached to the walls of the uterus and the two together constitute a kind of placenta (Balfour, op. cit.).

We may now proceed to a description of the various embryos obtained from gravid females:

1. The earliest stage found is that shown in Plate I, Figure 3. The large ellipsoidal yolk mass, having an axial length of 58 mm., is shown enclosed in the transparent egg capsule which had an overall length of 100 mm. The oval shaped blastoderm, measuring 29 mm. along its longer axis, lies on the posterior half of the yolk and at the rounded anterior end it shows the formation of the neural groove (ne.g.). The thickened edge of the blastoderm (b.e.) is well shown. The egg capsule is composed of an extremely thin membrane which is wholly transparent except at the polar regions where the capsule tapers to a sharply pointed end posteriorly and a blunter pointed end anteriorly. These ends are designated anterior and posterior in relation to the position of the embryo and not in regard to the position the capsule takes in the gravid uterus. The figure shows the capsule in the position in which it lies in the uterus, the blunter anterior end being directed posteriorly in relation to the female. The yolk has a thin covering of glairy albumen over the whole surface, but towards the poles the albumen is much thicker and becomes more or less opaque and is loosely attached to the capsule. Encapsulated eggs, which were removed from the uteri, were placed in a large pneumatic trough with running sea water. After a few days the capsules invariably ruptured around the equator and albumen was only found in the pointed polar ends. Compared with the egg cases of oviparous species, the egg capsules of *S. acanthias* contained very little albumen.

2. Plate II shows an egg capsule containing developing triplets (em. 1, 2, 3). These embryos were about 40 mm. in length, the total length of the capsule being

180 mm. The yolk was rounded in conformity with the shape of the enveloping capsule. All attempts to keep these embryos alive after the extrusion of the capsule failed. Embryo 2 ruptured its yolk sac and died, the remaining two embryos also succumbing in rapid succession.

3. The next stage is shown in Plate III, Figures 7A and B. These embryos were taken from a gravid female which had a single egg capsule in each uterus, each capsule containing a set of twins. Immediately after the female was opened, the capsules were removed from the uteri and placed in running sea water in a pneumatic trough. It was not possible to determine their age at this stage. After three days, one capsule ruptured and the embryos freed themselves from their enveloping membrane and lived for about twelve days. The twins in the other capsule died after five days and it is interesting to note that then they were smaller than their counterparts, although at the time of removal from the uteri, all the embryos were of equal size, viz., 45 mm. in length. The longitudinal diameter of the yolk sac at this time was 80 mm. The live embryos, a week later, had attained a length of 65 mm. The longitudinal diameter of the yolk sac was now 70 mm., thus showing a corresponding decrease as the embryo grows. The umbilical cord (u.c.) was relatively long and hollow, forming a tubular connection between the yolk sac and the intestine of the embryo. By a gentle pressure on the yolk sac it was possible to force particles of yolk along the umbilical cord into the embryo's intestine. Its walls were very thin and laterally in them ran the vitelline vein (v.v.) and artery. The internal anatomy was well developed at this stage, the alimentary canal being completely formed with a spiral valve developed in the valvular intestine. The liver was developed and the heart had assumed the typical Selachian form. The external gills (Figs. 8 and 9, e.g., sp.g.) had reached their maximum development. After twelve days the other pair died and the external gills were then beginning to shrivel up.

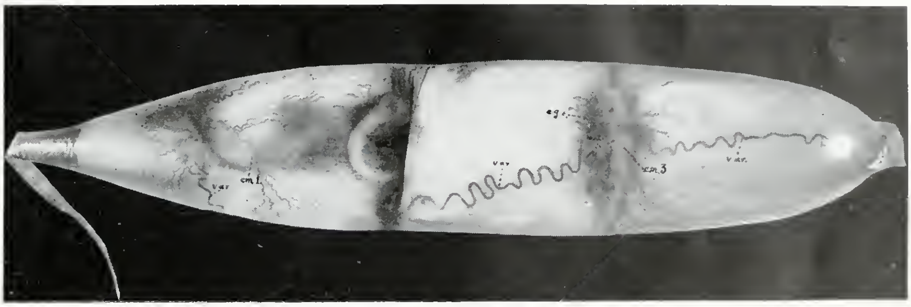

Plate II

Egg capsule containing three developing embryos.

4. A more advanced embryo is shown in Plate IV, Figure 12. Here the external gill filaments had disappeared. The yolk sac was now relatively small and the umbilical cord short and thick. The embryo had assumed the typical adult form except for the yolk sac.

5. The most advanced stages of development found in a gravid female occurred

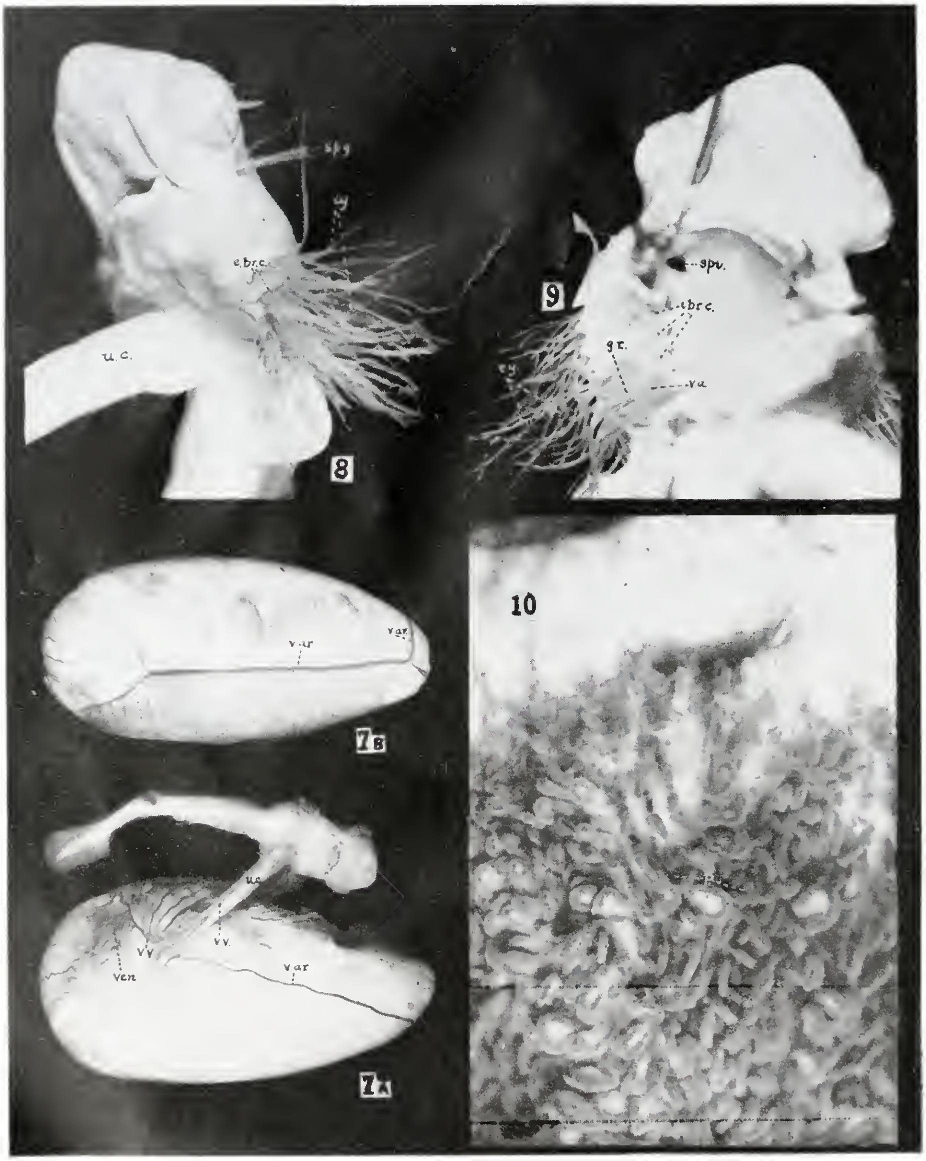

PLATE III

FIGURE 7A. A 70 mm. embryo showing the vitelline circulation and the attachment to yolk by the umbilical cord (u.c.).

FIGURE 7B. The hemisphere opposite to the one to which this embryo is attached.

FIGURE 8. Cephalic end of the same embryo showing the external gill filaments (e.g.) and the spiracular gill filaments (sp.g.).

FIGURE 9. Dissection of the buccal cavity of the same embryo showing the external gill filaments attached to the visceral arches (v.a.). The internal branchial clefts (i.br.c.) are also shown.

FIGURE 10. A portion of the internal surface of the uterus, much enlarged, showing the papillae (p.).

in a female 800 mm. in length. Here the six embryos were at the point of birth. They were 220 mm. in length and differed from the one shown in Plate IV, Figure 12 principally in the fact that the yolk had been completely resorbed and the remaining yolk sac only 5 mm. in length. The embryos had the same coloration as the adults. It was not possible to determine the age of these embryos so that up to the present the gestation period is unknown.

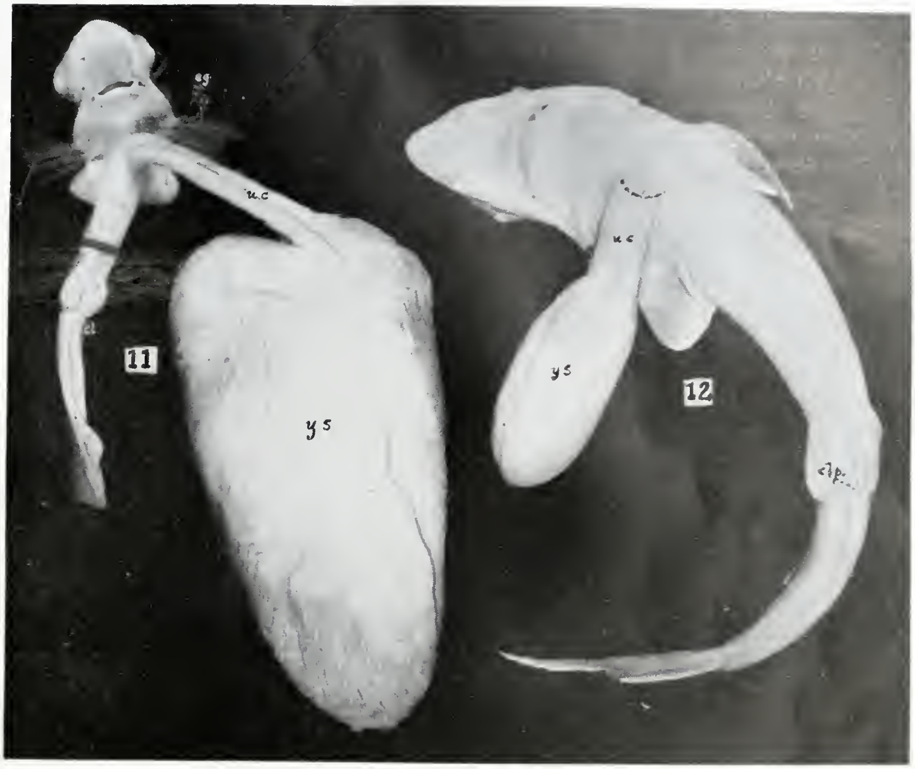

Plate IV

Figure 11. Entire embryo of 100 mm. attached to the yolk sac.

Figure 12. More advanced embryo 140 mm. in length. The dotted line at the proximal end of the umbilical cord (u.c.) shows the relative size of the yolk sac in a 220 mm. embryo.

Abbreviations

a. uterine artery, a.g. albumen gland, ap. apopyle, b.e. edge of blastoderm, c.d. closing device, cl. claw, clp. clasper, e.br.c. external branchial cleft, e.g. external gill filaments, em. 1, 2, 3. developing embryos, f.t. fallopian tube, f.t.o. coelomic orifice of same, g.r. gill rakers, hy. hypopyle, i.br.c. internal branchial cleft, l.ov. left ovary, m. mesentery, n.g. nidamental gland, ne.g. neural groove, ov. ovary, p. papillae, r. rhipidion, r.ov. right ovary, s. siphon, sp. spur, sp.g. spiracular gill filaments, spi. spiracle, u. uterus, u.c. umbilical cord, v. vagina, v.a. visceral arch, v.ar. vitelline artery, v.v. vitelline veins, ve. uterine vein, ven. venules, y.s. yolk sac.

EXTERNAL GILLS

Gudger (1940, pp. 626, 627) writing about external gill filaments in shark embryos states—"The embryos of all nonplacental viviparous sharks and rays known to me have long external gills. The eggs of these Elasmobranchs have thin diaphanous shells, through which uterine fluids readily penetrate. These fluids are milk-like secretions of the uterine mucosa and serve as food for the growing embryos, which absorb this food through their long filamentous gills. It has been indicated that the relatively thick shells of *Chlamydoselachus* are burst by the growing embryo, are cast off into the uterus, and are then or later thrown out into the sea. Two investigators (Hawkes, 1907 and Smith, 1937) have found highly vascularized areas in the wall of the right uterus (cf. my Plate III, Fig. 10). These observations suggest that these areas might have served to secrete foodstuffs into the uterus. Then the long gestation period and the enormous size of the relatively late embryos still attached to large yolk sacs seem to indicate that these embryos grow not at the expense of the yolk alone. All these things lead to the inevitable question—'Do the external gills of the embryos of *Chlamydoselachus* serve to absorb food from a uterine secretion?' . . . It seems quite sure that in any case these external gills of the non-extruded juvenile sharks serve as respiratory organs."

Further, Gudger (op. cit., pp. 559–562) in dealing with the matter of intrauterine gestation and respiration states (p. 561) "on the whole it seems quite probable that the young of both sharks (*Ginglymostoma* and *Chlamydoselachus*) may receive some oxygen by diffusion from the uterine wall into the fluids surrounding the embryo. Furthermore, from my knowledge of uterine gestation in other sharks and rays, I am strongly of the opinion that the uterine wall in both (sharks) secretes liquid food materials to nourish the young after they are freed from the egg capsules. . . . The long external gills of embryos of rays and of other sharks, when bathed in the uterine fluid, may take in not only oxygen but mineral salts and possibly other food substances as well. The rich plexus of vitelline capillaries will also be bathed in the fluid of the uterine cavity and they may absorb some food and oxygen from it. If this takes place in *Chlamydoselachus*, it must go on for a long time, until and even after the yolk is resorbed, and this yolk must be used up before the fish is born, else the free oceanic life of this little shark would be very brief."

There are many points of similarity between the present species and that described by Gudger. *Squalus acanthias* is ovoviviparous and at a certain stage of incubation the egg capsule bursts and the embryo is free to lie in the uterus. I have dissected gravid females in which parts of the capsule were found in the uterus. Plate IV, Figure 11, shows an embryo which lay completely free in the uterus with the remains of the capsule lying near the vagina, evidently ready to be extruded. The fact that it has been possible for me to keep some embryos of this species alive in sea water for some time after their removal from the uterus seems to indicate that the external gills must have a function purely respiratory. It is of interest to note that in all dissections of the uteri of gravid females the embryos lay with their heads pointed towards the cloaca and this leads me to infer that the relatively wide vaginal openings into the cloaca permit of sea water entering the uteri for the respiratory functions of the growing embryo. (Compare the provision of respiratory slits in the mermaid's purses of oviparous sharks through which water can enter to provide respiration by means of the external gills—von Bonde, op. cit.) The external gill filaments are doubtless precocious growths of normal gills and they are eventually lost during later development. Compare Figures 11 and 12 which portray embryos of greatly varying stages of development. In the latter figure the external gill filaments have completely disappeared and the functional gills are typically adult in structure. The occurrence of external gill filaments protruding from the spiracular cleft is conclusive evidence that the spiracular cleft in sharks was primarily a gill cleft with a respiratory function.

Widakowich (1907a) states that the external gills are lost as soon as the egg capular membrane disappears. This is contrary to my observations on embryos kept alive artificially in sea water, and also on those which had burst the egg capsule and were lying free in the uterus, but he does corroborate the view that these transitory gills do not serve the purpose of taking up nutrient material.

There is an alternative possibility in connection with the respiration of the embryo during the time that it has the external gill filaments. In no case has it been possible to observe any connection between the mucous lining of the uterus and the yolk sac even faintly resembling a placenta. This occurrence of a placental development in *S. acanthias* is much featured by Widakowich (op. cit.), but it seems more probable that the papillae of the uterus, which are richly supplied with blood vessels, can, by a process of osmosis, aerate the blood of the developing embryo through the capillary system in the external gill filaments. As mentioned before, Ercolani (op. cit.) grouped this species under his second group of uterine developments and he mentioned the fact that these papillae are in close contact with the vitelline membrane of the yolk. I am convinced, however, that at no stage during the intra-uterine development of the embryo does the mother play any part in the nourishment of the embryo by means of the external gill filaments, the yolk sac alone functioning in this capacity. This contention is, moreover, strengthened by the fact that the yolk sacs in all gravid females dissected by me never showed any convolutions into which the vascular papilla of the uterus could fit. There is of course a possibility that here also there may be an osmotic transference of blood from the surface vascular papillae to the vitelline circulation, but I cannot see that such a function is necessary to the developing embryo. I, therefore, lean to the view that the sole function of these vascular papillae is to provide aeration through the external gill filaments, this function being in addition to that previously described where such aeration takes place by means of the sea water entering the uterus.

The Vitelline Circulation

The earliest stages of the yolk sac circulation are shown in Plates II and III, Figures 7A and 7B. The vitelline blood vessels finally spread out over the whole yolk sac. Their function is to carry the nutrient yolk to the developing embryo. The early stages of the circulation were briefly described above. In all young embryos of *S. acanthias* a vitelline artery arises from the dorsal aorta and runs along the left side of the umbilical cord and thence forward from the yolk stalk (u.c.) over the surface of the yolk sac (v.ar.). In earlier embryos, this artery is sinuous (Plate II, v.ar.) but later it becomes straightened out. Its usual course runs from the umbilical cord to the anterior sharper apex of the yolk sac but there are variations where it runs obliquely across the yolk sac to the sides. In every case, however, it curves over the yolk sac and branches into two, one arm being short whilst the other passes straight back along the ventral surface of the yolk sac (Fig. 7B, v.ar.) and then forks into two branches. On this ventral surface the vitelline artery gives off numerous side branches at right angles to itself whilst no branches are seen arising on the dorsal surface of the yolk. Posteriorly, on the dorsal surface there is a single main vitelline vein (Fig. 7A, v.v.) which drains a dendritic group of venules (ven.). This main vitelline vein runs along the right side of the umbilical cord and enters the heart with the splanchnic vein. In its course along the umbilical cord the main vitelline vein is joined by smaller veins near the yolk sac.

SUMMARY

1. *Squalus acanthias* is an ovoviviparous dogfish with a wide range of distribution.

2. The male and female reproductive systems, especially the internal structure and the closing device of the uterus, are described.

3. Oviparous development takes place in a thin transparent egg capsule, different from the usual Selachian egg capsules, up to a certain stage, when the capsule ruptures and then the development becomes viviparous.

4. Embryos at different stages of development are described. Some capsules contained single embryos, others twins and still another triplets.

5. The functions of the external gills are dealt with, it being concluded that their function is primarily respiratory.

6. The vitelline circulation is described.

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EMBRYONIC DEVELOPMENT IN POECILIID FISHES

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In *Heterandria formosa,* a viviparous cyprinodont fish of the family Poeciliidae, nearly all of the nourishment for embryonic development is obtained from the mother after fertilization through a pseudoplacental association (Scrimshaw, 1944). Similar although less complicated associations between mother and embryo exist in other species of this family (Turner, 1937, 1940). These species are for the most part considered to be ovoviviparous. The distinction is based on the difference between live bearing forms which retain an egg with a full supply of nourishment for development and those whose embryos receive nourishment from the mother. The latter are considered truly viviparous.

Turner (1937) referred to all species of Poeciliidae as ovoviviparous. He obviously used this term in a general sense for he suggested in discussing the data of Bailey (1933) that *Xiphophorus helleri* could receive nutriment from the parent. Turner also pointed out (1937) that the small *Heterandria* egg cannot contain enough nourishment to account for the size of the larvae and suggested that the follicle cells surrounding the embryo furnish food materials.

The weight values reported by Bailey for various embryonic stages in *Xiphophorus* show no decrease in the weight of the total yolk-embryo system. Since energy is used for maintenance metabolism, the total weight of this system decreases in forms depending entirely on yolk. Gray (1928) reported a decrease of 37 per cent for the oviparous trout, *Salmo fario,* and Hsiao (1941) found a decrease of 34 per cent in the truly ovoviviparous perch, *Sebastes marinus.* Accordingly, although he does not suggest this, Bailey's data show that some nourishment must be obtained from the mother by the developing embryo of *Xiphophorus.*

It is true that most poeciliid fishes are more dependent on the yolk laid down before fertilization than upon maternally supplied nourishment after that time. However, the evidence presented below shows that the members of this family do utilize nourishment outside of that contained in the yolk and hence are not ovoviviparous in the strict sense of the term. Embryos of such species as *Heterandria formosa* and *Aulophallus elongatus* are truly viviparous and as dependent on the mother for nourishment as are those of a placental mammal.

Gray (1926, 1928) reported the relationships between nourishment and growth rate in the oviparous trout, *Salmo fario.* The problems of oviparity and ovoviviparity are similar in that in each the embryo has its own supply of nourishment and receives food and water from its environment. When these conditions are compared with true viviparity, striking differences are noted (Scrimshaw, 1944). The present study was undertaken to find and describe intermediate stages between viviparity and ovoviviparity in other members of the family. Such stages would aid in the understanding of the complex problems of viviparity in this specialized group. The weight changes in the egg and embryos of twenty additional species of Poeciliidae have been determined and a condition intermediate between ovoviviparity and true viviparity characterized. There appeared a remarkable tendency for the young of all the species examined to receive from the mother only as much in terms of dry weight as they required for the maintenance of tissues already formed.

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Material

Stocks of the following species were maintained in the laboratory and the gravid females were available as desired. These were dissected and the living embryos weighed.

Gambusia affinis holbrookii
Lebistes reticulatus
Poecilistes pleurospilus
Quintana atrizona
Xiphophorus helleri

The following species were collected in the field and the embryos weighed at the Institute for Research in Tropical America, Barro Colorado Island, Canal Zone (June–August, 1940).[2]

Allogambusia tridentiger
Aulophallus elongatus
Brachyrhaphis cascajalensis
Brachyrhaphis episcopi
Darienichthes dariensis
Mollienisia sphenops

In addition the writer is greatly indebted to Dr. Carl Hubbs of the Museum of Zoology, University of Michigan, for preserved material of the following species:

Allophallus kidderi
Belonesox belizanus
Brachyrhaphis terrabensis
Dextripenis evides
Gambusia nobilis nobilis
Mollienisia velifera
Poecilia vivipara
Poeciliopsis infans
Poeciliopsis occidentalis
Poecilistes pleurospilus
Pseudoxiphophorus bimaculatus taeniatus

Through the kindness of Dr. William Schroeder, additional specimens of *Gambusia affinis holbrookii* were obtained from the Museum of Comparative Zoology of Harvard University.

The gravid females of all of the above species were dissected and the contents of the ovary noted. In all of these forms the embryos are retained within the follicles of a large single median ovary which was removed intact before examination.[3] The embryos could be dissected out with the ovisac intact and the approximate diameter measured with an ocular micrometer. The weight determinations were made as described for Heterandria (Scrimshaw, 1944), the embryos being dried on small cover slips over calcium chloride and weighed on a Sartorius balance. Any error in the actual dry weight recorded was insignificant in comparison with the variability of the material. Wet weights are not discussed because of their variability in preserved material and the difficulties in determining excess fluid. The taxonomic scheme followed throughout is that proposed by Hubbs (1924, 1926).

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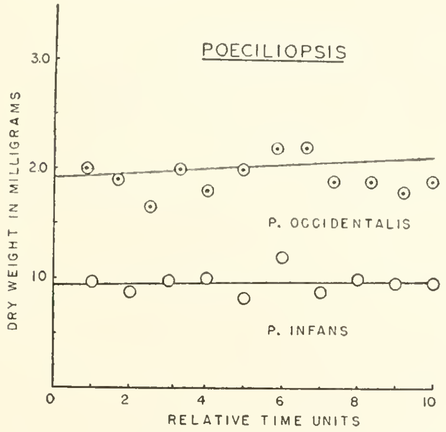
[2] This work was made possible by a grant from the Penrose Fund of the American Philosophical Society and the assistance of Dr. Thomas Barbour of the Museum of Comparative Zoology, Harvard University. The author was greatly assisted in the field work and weight measurements by Mr. Thomas C. Saunders of Harvard University. The cooperation of Mr. James Zetek, custodian of the island and laboratory; Mr. Graham Fairchild of the Gorgas Memorial Laboratory, Panama; and Mr. John Wood of the Canal Zone Police considerably aided this work.

[3] Dissection and weight data for all of these species are contained in the appendix of the author's doctorate thesis *Embryonic Growth in Poeciliid Fishes* deposited in the Harvard University Library.

EXPERIMENTAL

Dry weight determinations of the eggs and embryos in the intact ovisac were made at various stages between fertilization and parturition. In each case as many stages as could be obtained were studied and ranked according to age as described below for purposes of graphical and statistical representation.

It was soon observed that these weights were within the same general range regardless of the stage of the embryo. When least square lines were calculated through these points, a straight line parallel to the abscissa served to describe the data. Figures 1 and 2 show representative development lines and the scatter of weight values for four of these species.



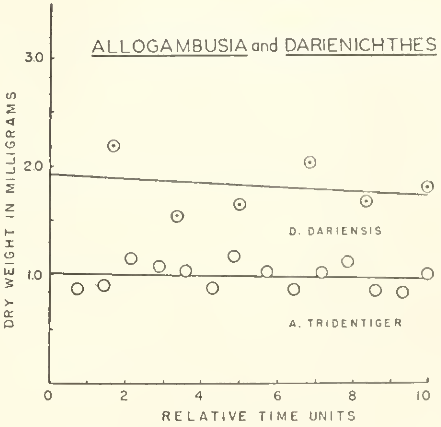


FIGURE 1. The dry weights of the embryos in the intact ovisac are arranged on a relative time axis in order of stage of development. For graphical and statistical purposes it has been necessary to assume that the time between stages is equal in all cases. Fertilization is represented at the beginning of the time scale and parturition at the end. The lines are calculated through these points by the method of least squares.

FIGURE 2. Two closely related species are portrayed in the same manner as those in Figure 1.

On the basis of these straight lines, the mean weight of the embryos of this group of poeciliid species can be compared (Figs. 3 and 4). The range is so great that all of the species could not be represented in one figure, but several forms are repeated in Figure 4 to facilitate comparisons. In addition to the species described in this paper, *Heterandria formosa* and *Aulophallus elongatus*[4] are included as examples of viviparous poeciliid fish securing nearly all of their nourishment from the mother. The straight line for *Aulophallus* is purely diagrammatic, but its use

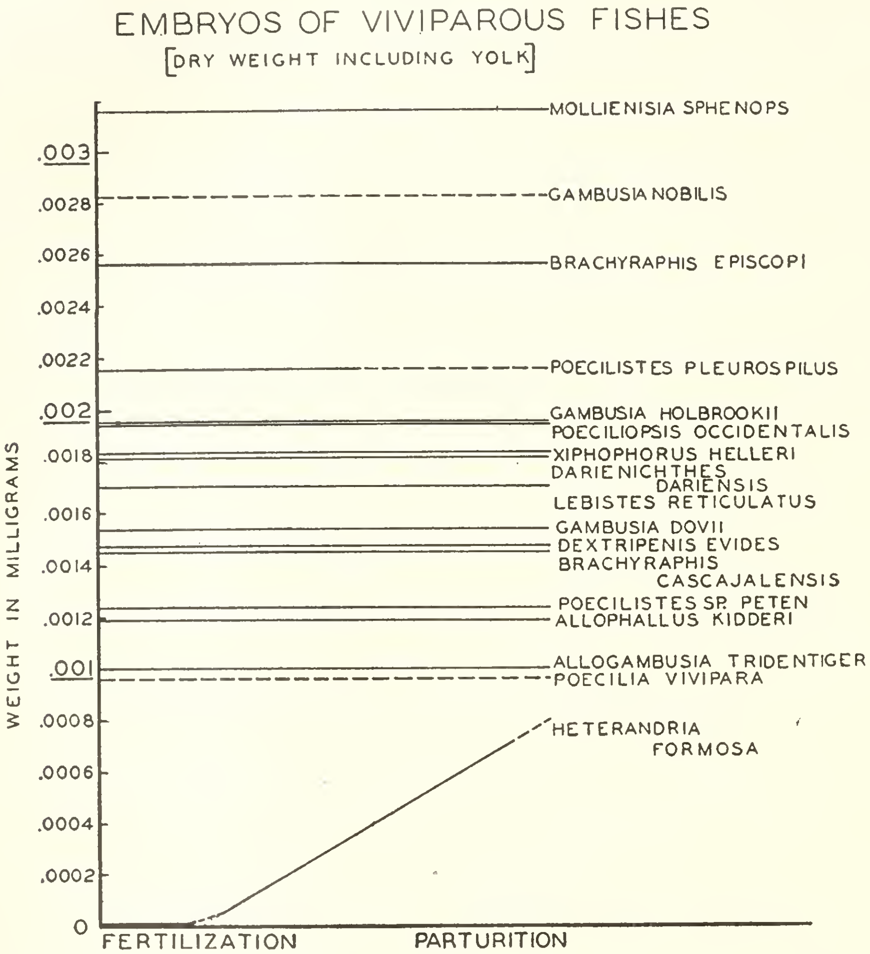


FIGURE 3. For purposes of comparison the dry weight curve of the embryo during development is drawn as a horizontal line. The average weight of all of the embryos of a given species is taken as the level at which the line is drawn. The degree to which these diagrammatic lines correspond to lines calculated through the data is discussed below. An approximation of the standard deviation observed from the above lines for each species is given by SE_y in Table I. *Heterandria formosa* is included as an example of a truly viviparous fish. The other species have previously been considered to be ovoviviparous. *Gambusia holbrookii* should be listed as *Gambusia affinis holbrookii*. *Poecilistes* sp. Peten has been identified by Hubbs as *Poecilistes pleurospilus* from Guatemala.

[4] Measurements on this species were also made in Panama during the summer of 1940. The weight of the fertilized egg and of the larva at parturition are indicated in Figure 1. A more complete discussion of this species is being prepared.

for *Heterandria* is probably legitimate (Scrimshaw, 1944). The broken lines indicate incomplete data. The known oviparous fishes have egg and larva weight greater than those of any of the fishes shown in Figure 1.

In Figures 1–6 no correction has been made for the effect of the fixative on the dry weight of the embryo. As can be seen from Figure 5, no single correction can be made for preserved material. Some embryos of *Gambusia affinis holbrookii* preserved since 1878 have changed very little in dry weight when compared with living ones studied in the laboratory. On the other hand, certain material collected in 1919 from the same place as the laboratory stock collected in 1941 has lost at least 70 per cent of its original dry weight. These specimens, however, were fixed in alcohol and preserved in strong alcohol (as high as 90 per cent). The 1878 material was fixed in formalin before it was transferred to the strong alcohol used as a preservative for fish by the Museum of Comparative Zoology. The material obtained from the Michigan Museum of Zoology was uniformly treated, having been fixed in 10 per cent formalin and changed to 70 per cent alcohol for storage.

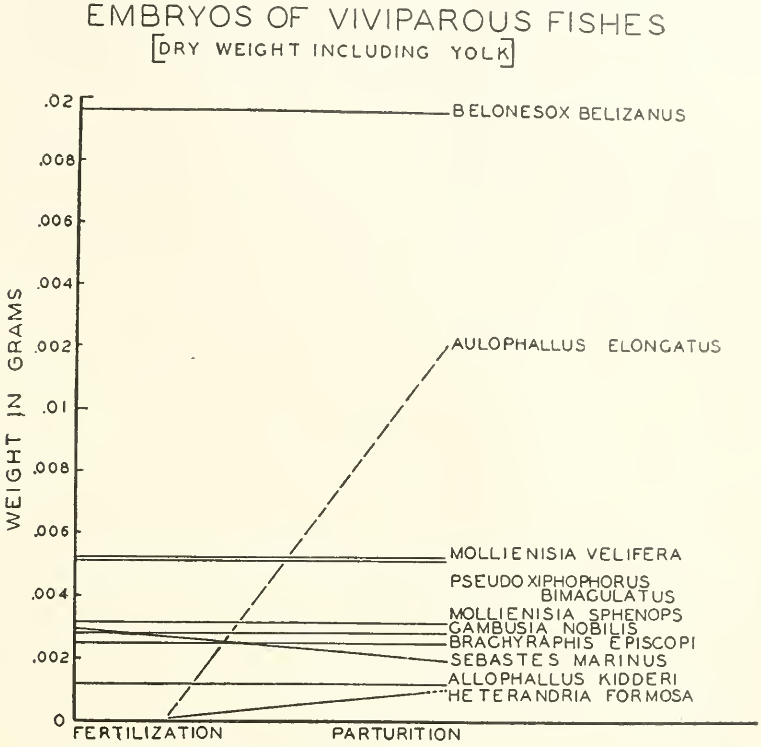
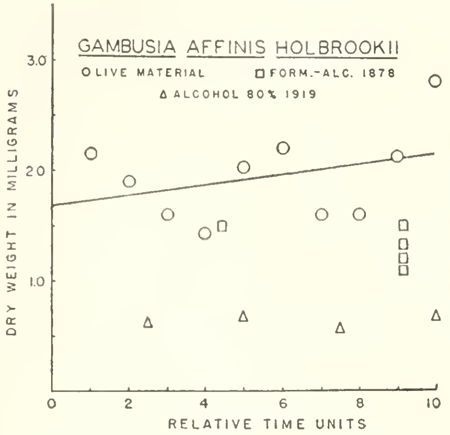


FIGURE 4. Additional species are shown in a manner identical to that described for Figure 3. The scale is enlarged to include species with greater embryonic weights. *Aulophallus* and *Heterandria* represent truly viviparous poeciliids. *Sebastes marinus* is a viviparous perch, a much larger fish than any of the others discussed and not a member of the Poeciliidae. It is included as an example of a truly ovoviviparous fish.

The dry weight of the embryo in fishes treated in this manner closely approximates that of the living form. The data plotted for *Brachyrhaphis cascajalensis* in Figure 2 illustrate this.

In Figure 4, the *Poecilistes* species has been identified by Hubbs as being a race of *Poecilistes pleurospilus* from Guatemala. The *Poecilistes pleurospilus* listed is laboratory stock obtained from the Everglades Aquatic Nurseries, Tampa, Florida. The marked difference in average weight of these two groups of specimens, assuming them to be the same species, is probably due to actual racial differences rather than to fixation. A similar situation was found for *Mollienisia sphenops*. Two forms which could be distinguished by size, coloration, and mean weight of the embryos were taken from Gatun Lake. The mean weight of the larger form is plotted in Figure 3 as 3.05 mg. The smaller forms showed a very constant embryo weight averaging 1.58 mg. Despite these differences, Hubbs (1942) was able to find no usual taxonomic character or measurement to justify considering the smaller form a separate species.



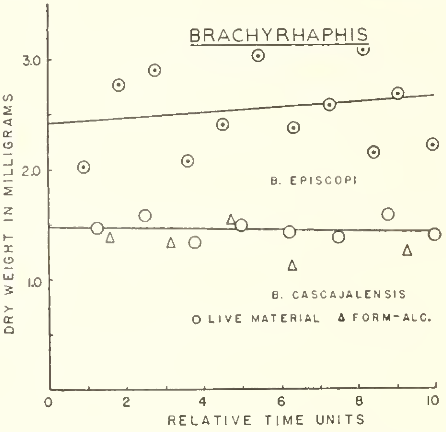


FIGURE 5. The dry weights of the embryo are plotted in a manner similar to Figure 1. The line is calculated from data represented by the circles. These represent embryos dissected out alive and dried immediately. The squares represent material preserved in formalin in 1878 and later transferred to the strong alcohol used in the Museum of Comparative Zoology, Cambridge, Mass. This varied between 75 and 90 per cent and was the sole preservative for specimens represented by the triangles.

FIGURE 6. The dry weights of the embryo are again plotted as in Figure 1. The triangles represent material preserved originally in 10 per cent formalin and later kept in 70 per cent alcohol. When weighed several months later there was excellent agreement with the values for living material studied at the time these were preserved. Most of the preserved material described has been handled in this manner.

Since the sperm in these fishes are viable in the ovary for several months, the time required to reach any given stage of development cannot be determined even in laboratory specimens. Therefore, in this study the embryonic weights were ranked in order of stage of development (cf. Scrimshaw, 1944). Since the data show that there is no consistent dry weight change during development, no error was introduced in the tabulation of the raw data. However, the assumption for graphical and statistical purposes of an equal interval of time between the stages studied means the use of a relative rather than an actual time scale.

In the figures presented, the first stage was taken to represent the weight at fertilization and the last stage the weight at parturition. The time between was arbitrarily divided into ten units. Thus in the case of *Brachyraphis episcopi* where ten stages were studied, each stage in Figure 6 is separated by an arbitrary relative time unit of 1.0. In the case of *Allogambusia tridentiger* represented by fourteen stages (Fig. 2), the ten point scale must be divided into fourteen parts. Thus each stage is separated by 0.71 unit.

Using relative time units as the abscissa and dry weight in milligrams as the ordinate, the raw data were recorded graphically. It was apparent by inspection that the data for each species could be represented by a straight line. Accordingly these were fitted by the method of least squares and were of the form $y = mt + k$. The intercept (k) of the straight line then represents the weight of the egg at fertilization. The slope (m) describes the weight change during development.

Table I

Species	N	k	SE_y	m	$m°$
1. *Gambusia affinis holbrookii*	10	1.676	.1127	.0502	2° 52′
2. *Darienichthyes dariensis*	6	1.925	.0584	−.0197	−1° 8′
3. *Poeciliopsis occidentalis*	12	1.923	.0456	.0027	0° 9′
4. *Poeciliopsis infans*	10	.937	.0108	.0046	0° 16′
5. *Aulophallus kidderi*	7	1.204	.0167	−.0040	−0° 13′
6. *Brachyrhaphis episcopi*	11	2.419	.1237	.0263	1° 30′
7. *Mollienisia sphenops*	12	3.045	.1495	.0204	1° 10′
8. *Dextripenis evides*	7	1.700	.0266	−.0350	−2° 0′
9. *Brachyrhaphis cascajalensis*	8	1.481	.0076	−.0055	−0° 19′
10. *Gambusia dovii*	10	1.667	.0479	−.0330	−1° 53′
11. *Allogambusia tridentiger*	14	1.005	.0132	−.0027	−0° 9′
12. *Xiphophorus helleri**	21	1.841	.1602	−.0009	−0° 3′
Total	118	Average .0034			0° 12′

N, number of embryonic stages; K, intercept of development curve in milligrams; SE_y, standard error or estimate of y in milligrams, $SE_y = \sqrt{\frac{\Sigma d^2_y}{N-1}}$; m, slope of development line; $m°$, deviation of development line from horizontal in degrees.

* Calculated from data of Bailey (1933).

As listed in Table I, the mean slope of all of these lines is only .0034. This is equivalent to an angle 0° 12′, an almost imperceptible deviation from a slope of 0. In order that these small deviations may be more readily visualized, the actual angle in degrees equivalent to the calculated slope is also listed in Table I. From inspection of the data, it can be concluded that the random variations from a slope of 0 can be accounted for by biological variation and sampling error. No significant change in the dry weight of the embryo in the intact ovisac can be detected during development.

A further statistical basis for the above conclusions can be obtained by placing the data for the different species in numerically comparable form.[5] This consists of adjusting the data already calculated to an arbitrary standard. The weight at fertilization (k) was made identical for each species and equal to unity (k_r). All other statistics were scaled accordingly by multiplication with the ratio $1/k$. The resulting curves ($y_r = m_r t + k_r$) are seen in Figure 7 and the new relative statistics are listed in Table II.

TABLE II

Species	m_r	$\hat{\sigma} m_r$	$t_{0.00}$	$t_{-.033}$	$P_{0.00}$	$P_{.033}$
1. *Gambusia affinis holbrookii*	.0300	.0213	1.409	2.961	.2	.02
2. *Darienichthyes dariensis*	−.0102	.0124	− .8270	1.841	.5	.15
3. *Poeciliopsis occidentalis*	.0014	.0068	.2032	5.028	.5	.001
4. *Poeciliopsis infans*	.0049	.0037	.7434	7.464	.5	.001
5. *Aulophallus kidderi*	−.0033	.0053	− .6324	5.653	.5	.001
6. *Brachyrhaphis episcopi*	.0109	.0154	.7049	2.845	.5	.02
7. *Mollienisia sphenops*	.0078	.0142	.5505	2.668	.5	.02
8. *Dextripenis evides*	−.0206	.0593	− .3473	.2093	.5	.5
9. *Brachyrhaphis cascajalensis*	−.0037	.0018	−2.044	6.088	.09	.001
10. *Gambusia dovii*	−.0198	.0091	−2.183	1.452	.05	.2
11. *Allogambusia tridentiger*	−.0027	.0035	− .7749	8.627	.5	.001
12. *Xiphophorus helleri**	−.0005	.0190	− .0268	1.709	.5	.1

k_r, common reference intercept, $k_r = 1.000$ (omitted from table, but essential part of data identical for all species); m_r, slope of relative development curve, $m_r = m \cdot 1/k$; $\hat{\sigma} m_r$, unbiased estimate of standard deviation of m_r; $t_{0.00}$, Fisher-t statistic, $t_{0.00} = \dfrac{m_r - 0.00}{\hat{\sigma} m_r}$; $t_{.033}$, Fisher-t statistic, $t_{.033} = \dfrac{m_r - (-.033)}{\hat{\sigma} m_r}$; $P_{0.00}$, probability that the difference in slope between the observed curve and one of zero slope is due to random sampling error; $P_{.033}$, probability that the difference in slope between the observed curve and one of − .033 slope (parturition weight = 2/3 fertilization weight) is due to random sampling error.

With the statistical measures for the 11 species adjusted in this manner, the impressions gained from the raw data can be evaluated. It was pointed out above that these fishes have been considered by most authors to be ovoviviparous. As will be discussed, this would mean that one-third of the nourishment originally available in the egg would be used before parturition for maintenance purposes. If this is so, the slope of a straight line development curve would be − 0.033 and the final dry weight would thus appear as ⅔ of the initial weight.[6]

The probability ($P_{-0.033}$) that the development curve of one of these species represents a chance deviation from a slope of − 0.033 can be determined by using the relation $t_0 = [m_r - (0.033)/m_r]$ and tables of the Fisher-t distribution. Examination of the $P_{-0.033}$ column in Table II shows that for most of the species examined the P is .05 or below. Thus, it is not probable that any of these curves represents a chance variation from a characteristic slope of -0.033. To conclude that this group of curves as a whole shows chance deviation from such a slope is impossible. It should be apparent that the lines in Figure 7 do not represent a group of ovoviviparous species.

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[5] The statistical data in Table II have been calculated by Mr. James Rafferty of the School of Medicine and Dentistry, University of Rochester. The statistical treatment of the data has been carried out entirely by him and the text description of the methods employed has been prepared under his direction.

[6] The slope of such a line would be − 0.33 if the units were equal for ordinate and abscissa. Since there are ten relative time units to a common reference weight of unity, the figure for the calculated slope becomes − 0.033.

However, is the apparent grouping of the development curves about a slope of 0.00 significant? The probability ($P_{0.00}$) that these lines represent chance variations from a characteristic slope of 0.00 can be found in a similar manner. It will be seen from Table II that the probability in most instances is .5 or better that this is the case. Taken as a whole, it is probable that the modal development curve for this group of species is a horizontal line. Thus the conclusion that there is no change in the weight of the embryo in the intact ovisac is substantiated.

The data for certain of the species require further comment. The data for species 2, 10, and 12 do show $P_{-0.033}$ values of .1 − .2. Of these, only species 10, *Gambusia dovii,* shows a real deviation from a line of 0 slope (Fig. 7). In the case of species 8, *Dextripenis evides,* with a $P_{-0.033}$ value of .5, there is enough initial variation in the weights that a deviation from a slope of 0.00 or -0.033 is equally probable.

It might also appear from Figure 7 that species 1, *Gambusia affinis holbrookii,* has pro-

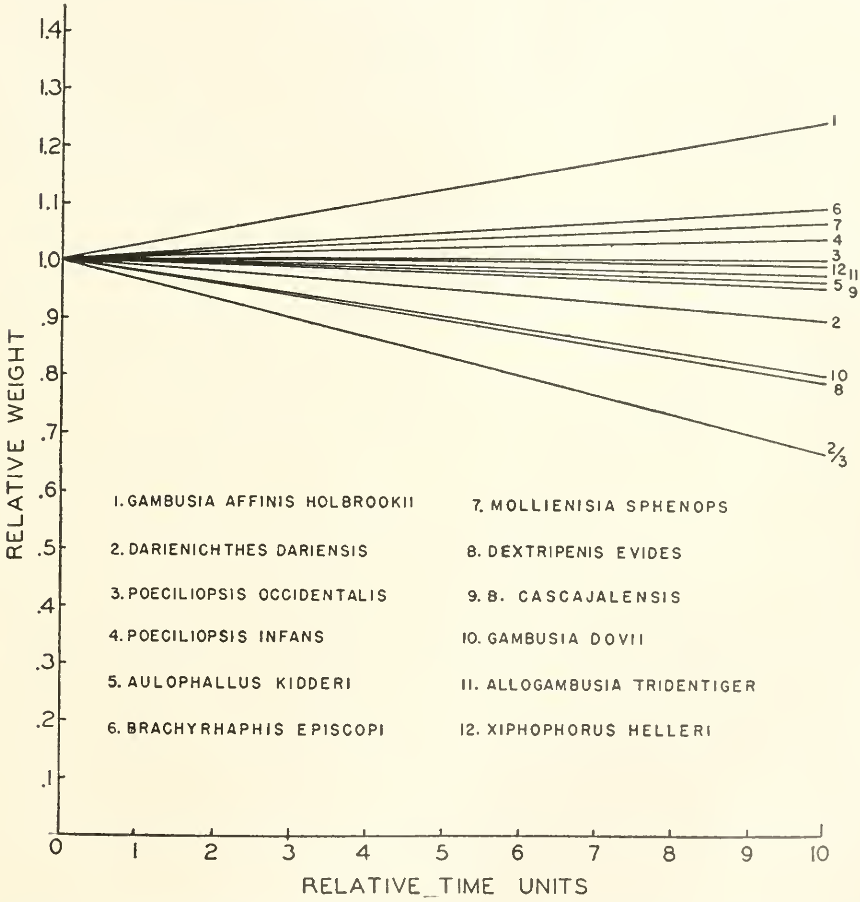

FIGURE 7. The weight data for the different species have been adjusted to an arbitrary standard so that the findings can be compared directly. The weight at fertilization (k) was made identical for each species and equal to unity (k_r). All other statistics were scaled accordingly by multiplication with the ratio $1/k$. Thus this new relative weight could be plotted as a function of relative time as used in the previous figures. The ⅔ line represents the line which the development curve of these species would follow if they were truly ovoviviparous.

gressed further than the other species toward true viviparity. While this possibility cannot be excluded, the P_0 value of .20 may be taken to indicate that this deviation from zero slope may be a chance occurrence.

In several species it was not possible to secure enough stages to establish the mean dry weight of the embryo or the change in dry weight with development. The limited data for these species are summarized in Table III. The three females of *Lebistes* studied represented pure inbred laboratory stock and the average embryo weight of all three widely separated stages proved to be 2.3 milligrams. It is probable that most or all of the species in this table would fit into the group represented in Table I if complete data were available.

TABLE III

Dry weight data for additional poeciliid species

Species	Number studied	Average weight (mg.)	σ	Adequacy of data
Belonesox belizanus	6	19.7	—	Range of 6.2–15.2 mg. for larva at parturition (6.2 value omitted from average)
Brachyraphis terrabensis	5	2.18	.81	Very narrow stage range and wide variation
Gambusia nobilis nobilis	8	2.8	.80	Only very early stages studied
Lebistes reticulatus	3		.0	Too few stages
Mollienisia vivipara	3	5.4	—	Few stages, very wide variation (3.4–6.3 mg.)
Poecilia vivipara	4	.96	.67	Too few stages
Pseudoxiphophorus bimaculatus taeniatus	5	5.3	—	Early stages only (Excessively low late stage value of 3.2 mg. omitted from average)

DISCUSSION

The variation in the weight of the intact ovisac at various embryonic stages seems to be a characteristic of the particular population of a species sampled. As described above, the standard error of y (SE_y) is actually a composite of errors. Nevertheless, it is primarily a reflection of the physiological and genetic variability to be found within the representatives of the species studied. It is probably valid to compare the inherent genetic variability of one species sample with that of another by means of this statistic. When the SE_y is small enough to minimize overlapping, the weight of the embryos should occasionally prove of taxonomic use.

The experimental data show the weight of the embryo at parturition to be the same as that of the egg at fertilization. Thus, quantitatively at least, the full amount of food material in the egg is converted into the substance of the embryo at the time of hatching. However, it is known that the food requirement of the embryo for maintenance metabolism during development is considerable.

Gray (1928) obtained for the plastic efficiency coefficient (P.E.C.) of the trout a figure of 0.63. This he believes to be a universal value for the synthesis of protoplasm. This coefficient is based on the dry weight of the yolk that can be accounted for by the dry weight of the fully developed larva. It represents the relative cost in weight units of building the tissues of the embryo. The higher the coefficient, the smaller the amount of burned substance in relation to the amount of nourishment initially available. Table IV summarizes the values for P.E.C. which have been reported. These values suggest that the mother must also contribute an amount of nourishment equal to about one-third the initial weight in the poeciliid fishes studied.

TABLE IV

Plastic efficiency coefficients of development

Species	Author	Coefficient
Trout (*Salmo fario*)	Gray (1926)	.63 embryo
Chick (*Gallus domesticus*)	Murray (1926)	.68 embryo
Frog (*Rana temporaria*)	Fauré-Fremiet & Dragoiu (1923)	.58 embryo
Silkworm (*Bombyx mori*)	Farkas (1903)	.59 embryo
Mould (*Aspergillus niger*)	Terroine & Wurmser (1922)	.59 normal growth
Viviparous perch (*Sebastes marinus*)	Hsiao (unpublished data)	.66 embryo
Trout (*Savelinus fontinalis*)	Gortner (1912)	.74 embryo (heat dried)

The maintenance cost of development is also illustrated by the various measures of the energy required during development. This subject has been well summarized by Needham (1931). Table V shows the values which have been obtained experimentally for one type of energy coefficient, the apparent energetic efficiency (A.E.E.), which gives the relation between the chemical energy in the fertilized egg and that combusted during development. For example, the figure cited by Tangl for the chick would indicate simply that 37.1 per cent of the original energy stored in the egg was lost by the time the yolk was consumed and the embryo well formed. All of the available evidence indicates that it is legitimate to generalize and to conclude that in the course of embryonic growth of animals developing from a yolked egg, about one-third of the energy initially available is expended for maintenance metabolism.

TABLE V

Apparent energetic efficiency of embryonic development

Species	Author	Efficiency
Chick (*Gallus domesticus*)	Tangl (1903)	62.9%
Chick (*Gallus domesticus*)	Murray (1926)	67.0
Fundulus (*Fundulus heterolitus*)	Glaser (1912)	52.8*
Frog to hatching (*Rana tempororia*)	Fauré-Fremiet & V. du Streel (1921)	82.0
Frog to end of yolk (*Rana tempororia*)	Fauré-Fremiet & Dragoiu (1923)	51.0

* This value was obtained by assuming that the same energy relationships prevailed for the use of the final half of the yolk as for the initial half. This figure is certainly too low.

The evidence that this relationship is also true for viviparous animals is more difficult to obtain. Rubner (1908) derived values, in part theoretical, for the efficiency of seven common mammals (horse, cow, sheep, pig, dog, cat and rabbit). The agreement of these values with the above data is sufficiently close to permit Needham (1931) to conclude that the real energetic efficiency (R.E.E.) of all embryos, mammalian as well as non-mammalian, is about .66. The R.E.E. involves a correction for basal metabolism, but for poikilothermic animals (and embryos such as those of the chick which are essentially cold blooded for the greater part of their prenatal life) it is so close to the A.E.E. that the two can be considered together.

An energy loss of about 34 per cent in the poeciliid fishes studied should be reflected in an appreciable decrease in the total amount of organic matter. The fact that no change in dry weight can be detected points to an exact correspondence between the weight of food used for maintenance and that of the nourishment supplied by the mother. But why should the majority of species in the family Poeciliidae stop at this identical point in the development of viviparity? Some similar factor or factors must be limiting the growth of the embryo to the initial weight of the egg in all of these species.

This might be the case if some essential factor in the yolk were present in a limiting quantity. If this factor could not be supplied by the mother, no amount of energy from her would permit the embryo to grow larger than the initial supply of this limiting substance would allow. Such might be the case if, for example, only simple sugars and certain amino acids were able to cross the placental barrier. Then certain of the essential amino acids, fats, vitamins or other factors might be available to the embryo in restricted quantities. However, the exact correspondence between initial and final weight in nearly all the species is difficult to understand on this basis.

In these fish the nourishment must pass through five to seven tissue layers: the maternal endothelium, maternal connective tissue (theca), maternal epithelium (follicular epithelium), possibly the chorion and vitelline membrane, yolk sac epithelium, embryonic endothelium, and probably some embryonic connective tissue. This situation resembles most closely the epitheliochorial type of placenta found in such animals as the pig. Despite the number of membranes in this type of placenta all of the materials necessary for growth and maintenance pass from the mother to the fetus across this barrier.

In some mammals, the materials most important for the elaboration of embryonic tissues, the amino acids, pass through the placenta against a concentration gradient in a manner suggesting a special mechanism (Needham, 1942). If a completely viviparous fish such as *Heterandria formosa* possessed such a mechanism, the steady gain in weight of its embryos can be understood. If, on the other hand, the fish discussed here lack such a mechanism, the failure of their embryos to gain weight might also be accounted for.

It is also of interest to note that carbohydrate is absent from eggs of oviparous fishes. Needham (1931), in summarizing the available data, expresses the belief that this is true because of the impossibility of supplying an oviparous form with sufficient sugar for embryonic metabolism without vastly increasing the size of the egg. However, there is no reason to believe that carbohydrate would not be used by their embryos in preference to the stores of fat and protein, if it were available. In the case of the mammalian fetus, Windle (1940, page 212) concludes from the available evidence that the fetus of the mammal practices a rigid glycogen economy at the expense of its mother's dextrose, drawing upon its own liver only in emergencies. It seems reasonable to expect that carbohydrate would also be the most readily available food which the embryo of the viviparous fish can obtain from the mother. Perhaps in the species discussed, carbohydrate from the mother exerts some sparing action on the tissue building materials of the yolk.

The discussion has been carried to this point to suggest something of the direct study which should be made on the functioning of the pseudo-placental barrier in these forms. The controlling mechanisms which are actually operating in the development of the species reported will not be clear until such experiments are carried out.

Summary

In poeciliid fishes the embryos are retained within the ovarian follicles until parturition. When the dry weight of the intact follicle is determined at various stages of development, no significant weight change can be demonstrated in the 18 species studied. This is in marked contrast to the situation in a truly ovoviviparous fish where one-third of the initial weight of the egg is used for maintenance during development. Accordingly, it is concluded that all of the species studied receive nourishment from the mother and hence are not ovoviviparous in the strict sense of the word. Furthermore, they apparently receive only as much nutriment, quantitatively at least, as they require for their maintenance metabolism. Some limiting mechanism must be operating, but its nature is not known. This situation is contrasted with that in *Heterandria formosa* and *Aulophallus elongatus,* members of the family which have developed nearly complete dependence on the mother for nourishment during development.

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THE EFFECT OF TEMPERATURE ON THE WINGS OF DIMORPHOS/DIMORPHOS VESTIGIAL-PENNANT/VESTIGIAL IN DROSOPHILA MELANOGASTER

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Harnly (1930b) and Stanley (1931) showed a critical temperature for the increase in wing size of the homozygous vestigial flies. This response is direct but not proportional to the rise in temperature. It was found that the introduction of the sex-linked modifier dimorphos into this genotype lowered the critical temperature for wing enlargement 5° C. for the males and 2° C. for the females (Harnly and Harnly, 1935). Again the response was direct but not proportional throughout the viable temperature range. Vestigial-pennant is a reverse mutation from vestigial to a recessive wild type allele. The wings of these flies showed an inverse response to temperature, and a critical point appeared to occur between 28° and 30° (Harnly and Harnly, 1936). It was also found that the heterozygous vestigial-pennant/vestigial wings showed an inverse response to temperature from 16° to 22° and a direct response from 26° through 32°.

These shifts in critical temperatures, the direct or inverse response of wing size to temperature of the homozygotes, and the apparent reversal of dominance with temperature in the heterozygote led to this study of the dimorphos/dimorphos vestigial-pennant/vestigial genotype.

Stocks and Methods

Through a knowledge of the phenotypes produced by dimorphos vestigial, homozygous vestigial-pennant and the heterozygous vestigial/vestigial-pennant at 22° and 25°, it was possible to develop a stock of dimorphos vestigial-pennant. Isogenic stocks of dimorphos vestigial and dimorphos vestigial-pennant were employed in these experiments. Due to the breeding technique (Harnly and Harnly, 1935, 1936) these stocks differ from each other in genotype only at the vestigial locus.

The culture methods, procedure for measurement of wings, etc. were the same as those described in earlier papers of this series (Harnly, 1932, 1936; Harnly and Harnly, 1935). Homozygous dimorphos vestigial-pennant females were mated to homozygous dimorphos vestigial males. Their offspring (dimorphos/dimorphos vestigial-pennant/vestigial) were allowed complete development at 16°, 18°, 20°, 22°, 24°, 26°, 28°, and 30° C. Those F_1 individuals placed at 32° for development were returned to a 26° incubator after the completion of five days (120 hours) of development and there completed their growth and their emergence.

Results

Wing length

The genes used and their symbols are: vestigial (vg), vestigial-pennant (vg^p) and dimorphos (di). The curves for the mean wing length for the di/di vg^p/vg

males and females show an inverse response to temperature increases from 16° to 28°, and are similar in slopes and values to those previously recorded for the wild type (Stanley, 1935) and homozygous vg^p (Harnly and Harnly, 1936). The mean wing lengths in mm. of di/di vg^p/vg males and females are shown in Table I.

TABLE I

Mean wing length in mm., di/di vg/vg^p., 5 trials

° C.	Males				Females			
	Length	±	P.E.	σ	Length	±	P.E.	σ
16°	2.53	±	0.0097	0.1241	2.60	±	0.0089	0.1135
18°	2.49	±	0.0056	0.0724	2.59	±	0.0103	0.1306
20°	2.42	±	0.0055	0.0707	2.53	±	0.0086	0.1101
22°	2.32	±	0.0082	0.1030	2.43	±	0.0119	0.1524
24°	2.25	±	0.0069	0.0897	2.33	±	0.0121	0.1550
26°	2.25	±	0.0066	0.0755	2.35	±	0.0109	0.1257
28°	2.11	±	0.0073	0.0939	2.27	±	0.0094	0.1205
30°	2.15	±	0.0067	0.0859	2.28	±	0.0067	0.0864
32°	2.24	±	0.0081	0.0969	2.32	±	0.0069	0.0886

The di/di vg^p/vg males and females show a gradual decrease in wing length from 16° to 28° beyond which there is a gradual increase in wing length to 32°. Apparently 28° is a critical point since the slope is negative below this temperature and is positive above it. The female wings are consistently longer than those of the males throughout the temperature range. The similarity in slopes and values of the di/di vg^p/vg male and female curves indicates there was no difference in the response of the two sexes in wing length over the range employed.

Wing area

The mean wing areas for di/di vg^p/vg will be found in Table II and are shown

TABLE II

Mean wing area in sq. mm., di/di vg/vg^p., 5 trials

° C.	Males				Females			
	Area	±	P.E.	σ	Area	±	P.E.	σ
16°	1.591	±	0.0090	0.1451	1.327	±	0.0171	0.2161
18°	1.555	±	0.0071	0.0914	1.171	±	0.0171	0.2168
20°	1.482	±	0.0086	0.1106	1.065	±	0.0151	0.1934
22°	1.338	±	0.0086	0.1080	0.964	±	0.0156	0.2006
24°	1.278	±	0.0219	0.2313	1.063	±	0.0200	0.2568
26°	1.267	±	0.0064	0.0730	1.111	±	0.0163	0.1867
28°	1.138	±	0.0080	0.1026	1.219	±	0.0116	0.1492
30°	1.214	±	0.0062	0.0796	1.294	±	0.0065	0.0840
32°	1.291	±	0.0080	0.0957	1.342	±	0.0093	0.1192

in Figures 1 and 2 together with those of Harnly (1930b) and Harnly and Harnly (1935, 1936) for the genotypes indicated.

The male curves for vg^p and di/di vg^p/vg coincide and have a negative slope between 16° and 28°. Between 28° and 32° they diverge sharply, the homozygote having a higher negative value while the heterozygote has a definitely positive slope. Apparently 28° is the critical point for length and area in the males of both genotypes.

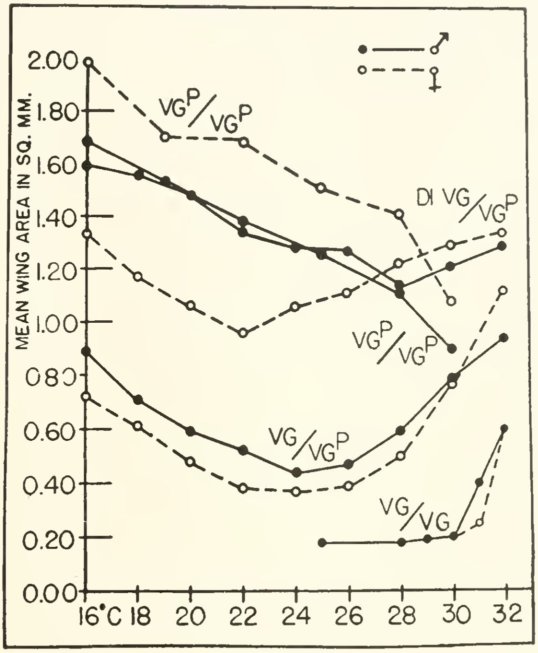

Figure 1.

The curve for the di/di vg^p/vg female wing area is U-shaped as is that of vg^p/vg females between 16° and 32°, but their wings are approximately double the area of the vg^p/vg wings. From 16° to 22° there is a gradual decrease and from 22° to 32° there is a steady increase in the area of the di/di vg^p/vg female wings. The reversal in the sign of the slope of the curve at 22° demonstrates that this is the critical temperature for the di/di vg^p/vg females. Between 26° and 28° the female curve crosses the male curve indicating a larger wing area for the females beyond that point up to 32°.

Shift in Wing Form Threshold

The male wing phenotype did not vary significantly from 16° to 32° in the di/di vg^p/vg flies. However, this genotype appears to be responsive to temperature, in that the percentage of perfect wings varied with the temperature. Wings with minor nicks were produced at all temperatures, but perfect wings were only produced at 16° (19%), 18° (11%), 26° (10%), 28° (7%), 30° (7%), and 32° (28%).

The original homozygous vg^p stock showed only occasional nicks in the distal margins of the wings (Harnly and Harnly, 1936). Repeated back crossing of the vg^p to the inbred vg stock has introduced one or more wing margin genes intensifying this tendency. This resulted in a vg^p stock that regularly showed minor nicks at all temperatures, but produced perfect wings at 16° (Harnly and Harnly, 1936). Due to their origin the homozygous di vg^p and the homozygous di vg stocks presumably carried the same wing margin gene or genes for the production of these minor nicks or notches.

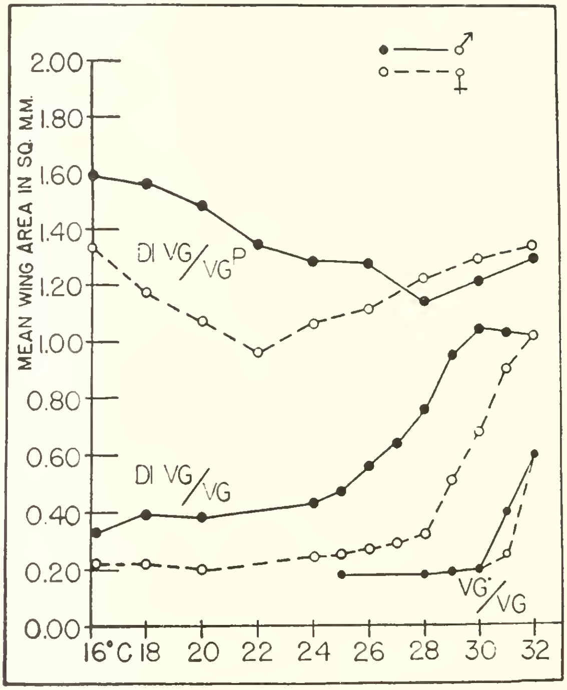

Figure 2.

The variation in frequency of nicks through the visible temperature range indicates that the wing-margin gene or genes are thermolabile at some period during the development of the individual. The largest number of perfectly normal wings appear at the temperature extremes, namely 16° and 32°. The smaller percentages of normal wings are found at temperatures close to the upper extreme. This indicates that some genes affecting wing margins are expressed at low temperatures while others find expression at high temperatures. Harnly (1942) obtained parallel results from reciprocal crosses between the two vg^p stocks and between these and a new vg^p stock. The di/di vg^p/vg genotype evidently produces full wings in the males with the possibility of minor variations at the distal end through the action of these wing-margin genes.

The di/di vg^{p}/vg female wings offer an entirely different picture. From 16° through 26° the phenotype varied around over-sized "antlered" with the occasional appearance of a "strap" wing. At 24° and at 26° phenotypes resembling the vestigial alleles antlered, snipped, carved and notched also appeared; from 28° to 32° the wings were predominantly "notched" to "nicked." Perfect normal wings appear only at 32° and 24 per cent of these wings were normal.

The di/di vg^{p}/vg female wings at 16° were similar to the male wings of the vg^{p}/vg described by Harnly and Harnly (1936) at 16°. The introduction of di into the vg^{p}/vg genotype has lowered the threshold for the wing pattern sequence previously described (Harnly and Harnly, 1935). This results in a pattern grade (antlered) for the female at 16° the equivalent of that first observed between 28° and 30° in the genotype (female vg^{p}/vg) carrying the normal allele of this sex-linked modifier (Harnly and Harnly, 1936). At these higher temperatures the di/di vg^{p}/vg females graded phenotypically "notched" to "nicked." The di gene had lowered the pattern threshold some 12° in the females and had lowered it so far in the males that only the full-wing pattern grade was produced throughout the viable temperature range.

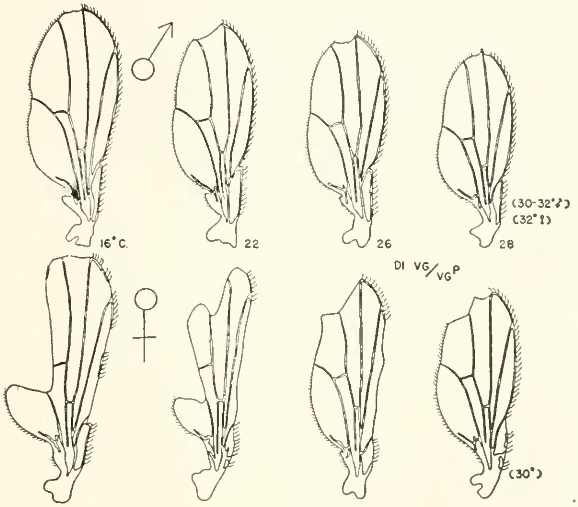

Figure 3.

Shifts in the Critical Temperature

The di mutation arose in an inbred homozygous vg stock and consequently the di vg genotype differed from this inbred vg strain only in the allele present at the di locus. The original vg^{p} mutant arose in a culture of black vestigial flies. This mutant had been backcrossed for many generations to the inbred vg stock. This produced a vg^{p} line differing from the inbred vg strain by only the allele at the vg locus. Therefore, these three genotypes differed only by known alleles at the vg and di loci. Consequently the introduction of di into this backcrossed vg^{p} stock produced a change in only one locus. The experiments on vg, di vg, vg^p/vg and vg^p (unpublished) have been reported previously (Harnly, 1930, 1936; Harnly and Harnly, 1935, 1936). Including our own experiments on di/di vg^p/vg truly comparable data is now available on five isogenic genotypes.

The di/di vg^p/vg male wings are phenotypically wild type throughout the temperature range employed. However, as mentioned above, "nicks" occasionally appear at the distal margins of the wings. These "nicks" are due to wing-margin genes (Harnly, 1942). The curves for male wing length and wing area coincide with the wild and vg^p curves and demonstrate a similar response to temperature between 16° and 28°. The reversal in the sign of the slope of the di/di vg^p/vg curves between 28° and 32° is markedly contrasted with the further fall of the wild and vg^p curves. This response is due to the combined action of the di and vg genes as will be shown later. The female length curve of di/di vg^p/vg parallels that of the males throughout the temperature range. Evidently under the conditions in these experiments, the threshold for the wild phenotype and temperature reactions of the male and the length response for the female has been reached in this genotype.

The critical temperature is a function of the genotype. The response of vg is direct with temperature and the value of the slope of the curve increases sharply above 30°. The di gene lowers this critical temperature 2° in the homozygous vg female. The slope of the wing area curve of homozygous vg^p females is negative in sign. The wing area curve of the heterozygote vg^p/vg has a negative slope from 16° to 24° and a positive slope from that point to 32°. The area curve of the di/di vg^p/vg females has a negative slope from 16° to 22°, and a positive slope from there to 32°. Again, the introduction of the dimorphos gene has lowered the critical temperature 2°, in the females.

These shifts in the critical temperature from genotype to genotype may be explained as the result of changes in dominance. On that basis the critical temperature (e.g. 24° for vg^p/vg) is the point at which a reversal in dominance occurs between the alleles vg^p and vg in the heterozygote as was suggested earlier by Harnly and Harnly (1936). The data from these experiments with di/di vg^p/vg indicate that there may be two critical temperatures: (1) the reversal of dominance as indicated in the wing area curves; and (2) the phenotype threshold (discussed above), a problem of differentiation as opposed to that of growth in development. It may be that further work will show that both of these are different manifestations of the same phenomenon.

SUMMARY

1. The genotype di/di vg^p/vg was examined at 2° intervals from 16° to 32°.

2. The length of the wings on the males and the females decreased from 16° to 28° and increased from 28° to 32°. The wings of the females were longer than those of the males at all temperatures.

3. The area of the wings of the males decreased from 16° to 28° and then increased to 32°.

4. The critical temperature for both the length and area of the male wings is apparently 28°.

5. The area of the wings of the females decreased from 16° to 22° and increased from 22° to 32°. The resulting U-shaped curve is very similar to that reported

previously for vg^p/vg but the values are approximately double for the $di/di\ vg^p/vg$ genotype.

6. The critical temperature for the female wing area appears to be at 22° and for wing length at 28°.

7. The di gene lowers the critical temperature for wing area 2° in the vg/vg female and the vg^p/vg female.

8. The critical temperature is a function of the genotype.

9. The males have wild type wings with minor marginal nicks at all temperatures. Wings with perfect margins were produced at 16° (19%), 18° (11%), 26° (10%), 28° (7%), 30° (7%), and 32° (28%). The marginal defects are obviously thermolabile.

10. The wings of the females varied around over-sized "antlered" with the occasional appearance of a "strap" wing from 16° to 26°. Phenotypes resembling the mutant alleles antlered, snipped, carved, and notched appeared at 24° and 26°. The wings were predominantly "notched" to "nicked" from 28° to 32°. Perfect margins were observed on twenty-four per cent of the wings developed at 32°.

11. The di gene lowered the wing pattern threshold some 12° in the females. In the males, this threshold was lowered below the viable range and only normal wings were produced.

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SUBSTRATE-ENZYME ORIENTATION DURING EMBRYONIC DEVELOPMENT [1]

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Introduction

Since Godlewski's (1900) observations it has been known that the rate of respiration increases during the course of amphibian development. Numerous workers (cf. Moog, 1944) have confirmed these results with more accurate measurements. The work of Loeb (1895) and Philips (1940) on Fundulus embryos and Brachet (1934) on amphibians made it evident that a rigid causal dependence of embryogenesis on oxygen consumption does not obtain in all instances. These authors found that a considerable portion of early embryonic development could proceed in the complete absence of oxygen.

Relatively few attempts have been made to analyze experimentally the increase in oxygen consumption during development. Such an increase could presumably involve: (1) an increase in the permeability of the eggs to oxygen during development, (2) synthesis or activation of more enzymes, (3) formation of additional substrate, and (4) reorientation of enzyme and substrate, initially present, but functionally disconnected. This could be accomplished by a spatial separation between the two and the immobilization of a necessary carrier.

The first mechanism is made unplausible by the findings of Parnas and Krasinska (1921), confirmed by Brachet (1934), who found the respiratory rate of amphibian embryos to be, within wide limits, independent of oxygen tension up to neurulation. During this same period a 4 to 5 fold increase in rate of oxygen consumption is realized.

It seemed probable that a systematic comparison of the respiration of brei and intact eggs during development combined with an examination of the cytochrome oxidase content could provide data which would permit a decision amongst the last three hypotheses mentioned. The present paper presents the results obtained from such experiments. They support the hypothesis that control of respiratory rate in developing embryos is effected by a spatial orientation of enzyme and substrate. No detectable increase in the cytochrome oxidase content was observed and the substrate content decreases rather than increases during development.

Materials and Methods

Eggs

The eggs of *Rana pipiens* were obtained after injection of anterior pituitary gland. They were artificially fertilized and after swelling of the jelly were cut up into small groups in 10 per cent Ringers solution and kept at 20° C. The stages at which the eggs were selected for measurements were determined according to the schedules of Pollister and Moore (1937) and Shumway (1941). Where desirable intermediate stages were devised with the aid of photographs published in Rugh's (1941) manual.

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In preparation for an experiment, the eggs were plucked clean of their jelly with the aid of a pair of fine forceps and filter paper. The denuded eggs were immediately placed in the M/15 phosphate buffer at pH 6.5. The number of eggs used in each flask varied at different stages. The attempt was made to use enough material to give ca. 5 mm. movement of the manometer fluid for each ten minute reading. The eggs were suspended in sufficient fluid so that the total volume was 2 cc. and transferred to the respirometer vessel.

Manometric measurements

All measurements were carried out at 26.2° C. in Barcroft-Warburg Manometric respirometers. The vessels were provided with side arms for substrate additions. The rate of shaking was 100 complete oscillations per minute with an amplitude of 8 cm.

Preparation of brei

After some preliminary experiments the following was adopted as the most satisfactory available method for obtaining breis of the eggs: The correct number of eggs was diluted to 20 cc. with chilled M/15 phosphate buffer and the suspension placed in a Waring blender. This volume is sufficient to almost cover the blades. When for various reasons less volume was used, tilting of the blender was resorted to during its operation. The blender was turned on intermittently until complete destruction of cell structure was attained as determined by direct microscopic observations. Care was taken to keep the temperature of the fluid below 25° C. during the process. Aliquots of the cell-free suspension were then dispensed to the vessels.

It may be noted that attempts to prepare cell-free breis by grinding with purified sand invariably led to considerable inactivation of the respiratory enzymes as evidenced by the consistently lower rates of oxygen consumption of such preparations as compared with those made with the blender. We had no success with the violent shaking procedure described by Brachet (1934). Fewer than 20 per cent of the embryos were disrupted by this method which in Brachet's hands yielded almost 100 per cent cytolysis. This difference may be attributed to a relatively greater sensitivity of the European species (*Rana temporaria*) to mechanical disruption.

The respiration of the breis starts to decline after about 60 minutes. Consequently rates were determined in the earlier constant rate portion. All values reported were obtained by measuring the slope of the straight line from 0 to 60 minutes.

Cytochrome oxidase

Cytochrome oxidase was measured following the precautions noted by Stotz (1939). In excess of 10^{-4} mM. per cc. of cytochrome C prepared by the method of Keilin and Hartree (1937) from beef heart was included with each suspension in measuring cytochrome oxidase activity. As substrates, paraphenylenediamine (ppd) hydroquinone and adrenaline were employed in concentrations of 1/50th molar. In the case of the latter two, a correction for autoöxidation was necessary. This was done by including one flask in which all reagents were used as before except that the cell extract was placed in boiling water for 5 minutes. The readings so obtained were subtracted from those of the other flasks. Such corrected readings did not differ from those obtained with paraphenylenediamine which has a negligible rate of autoöxidation. Care was taken to keep the tissue content per flask below levels which would yield respiratory rates exceeding 600 cu. mm. per hour when substrate was added.

Within 30 minutes of the addition of substrate from the side arm, a very sharp drop in the rate of oxygen consumption is observed. Five minute interval readings were taken after the introduction of substrate and the calculation of cytochrome oxidate activity is based on the slope of the straight obtained by plotting the oxygen consumption against time in the 10–20 minute period following the addition of substrate. All the cytochrome oxidase activity measurements on the breis were performed within 60 minutes of their preparation.

Experimental Results

Using the methods described, a detailed comparison was made of the respiratory rates of breis and whole eggs during the course of development. The results obtained are summarized by Figure 1.[3] The general exponential nature of the curve describing the variation of respiratory rates of intact embryos during development agrees with those recently published by previous authors (Atlas, 1938; and Moog, 1944). An exception may be noted in the data reported by Barnes (1944) in which a linear rather than exponential rate of rise is observed between stages 12 and 17. Barnes does not discuss this discrepancy between her results and those published earlier by Atlas.

It may be noted in passing that in agreement with Atlas (1938) and Moog (1944) the curve for intact cells in Figure 1 does not rise continuously. A break occurs between stages 12 and 13, a little later in development than the discontinuities observed by the other authors.

The respiration of the brei is high from the very earliest stages of development and remains so well into stage 13 which corresponds to the onset of neurulation. Beyond this stage however it starts to fall, remaining however above the rate of intact eggs in corresponding stages until about stage 17. Beyond stage 18 the respiration rates of the breis are definitely below those of the corresponding embryos. These results confirm the observations of Brachet (1934) who noted that cytolysis of the eggs of *Rana temporaria* during early embryogenesis led to marked increases in the rates of oxygen consumption whereas later in development decreases always followed cytolysis.

It is quite apparent from the respiratory rates of the breis that the eggs contain from the very onset of development sufficient enzymes and oxidizable substrate to support a much higher rate of oxygen uptake than the intact embryos actually do.

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[3] The authors are deeply indebted to Mrs. Helen Spiegelman for her able assistance in making the many calculations.

From this result alone it would seem unnecessary to postulate the formation of additional enzyme or substrate to account for the rising respiratory rate during development. The early high rates of the breis can be explained by assuming that the destruction of the cellular structure permits a freer contact of substrate with enzyme than occurs in the normal embryo. The fall in respiration of the brei observed beyond stage 13 can also be explained on the same basis. With the passage of time more and more substrate is consumed resulting in a depression of the brei respiratory rate due to substrate, or possibly carrier, dilution.

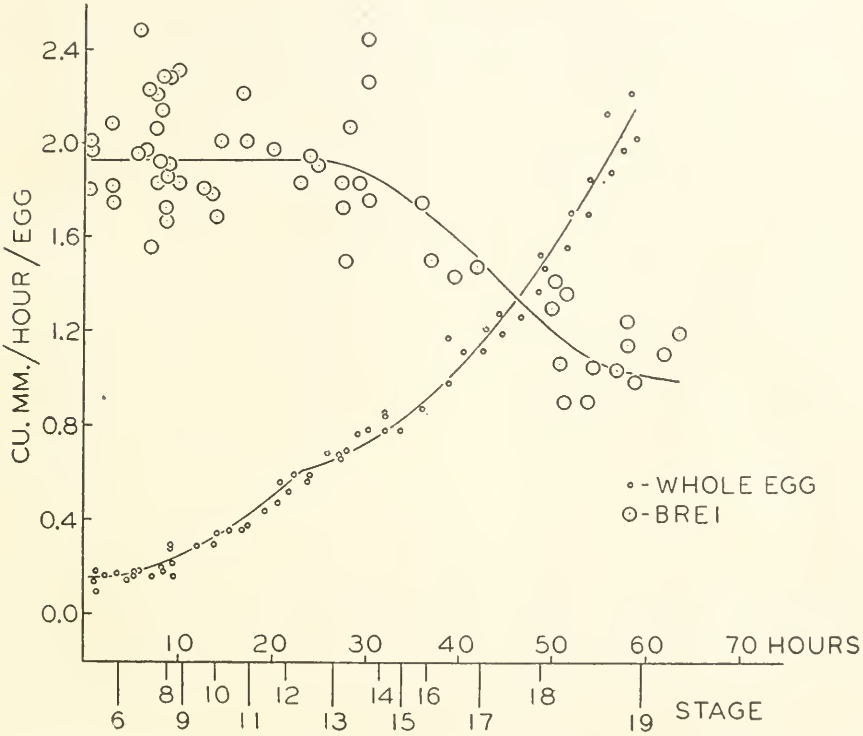

FIGURE 1. Comparison of respiration rates of brei and whole embryo (egg) during the course of development.

Response to cytochrome C

While it was not expected that externally placed carriers could penetrate into the cells, experiments were performed in which very heavy concentrations of cytochrome C, up to 2 × 10^{-3} mM. per cc., were placed in the external media in which embryos in early stages were suspended. No effect on respiratory rate could be detected.

Since the fall in brei respiration beyond stage 13 might possibly be due to a decrease through loss or inactivation of cytochrome C, this substance was added to breis prepared at various stages of development. Control readings were taken for approximately ½ hour before transferring sufficient cytochrome C from the side arms to the main compartment to give a concentration of at least 10^{-4} mM. per cc. This concentration was chosen since it gave maximum rates when substrates of the cytochrome oxidase system were also added. The results are summarized in Table I. It is evident from this table that no significant changes occur on adding cytochrome C. Thus brei respiration is not limited by the content of this factor and the fall in rate beyond stage 13 cannot be ascribed to variations in its concentration.

TABLE I

The effect on respiration rate of adding cytochrome C to brei prepared at different stages of development. All values are based on the cu. mm. of O_2 consumed/egg/hour.

Stage	Respiration before addition of cyt. C	Respiration after addition of cyt. C	Change
3	1.91	1.90	−0.01
5	2.20	2.10	−0.10
6	1.85	1.90	−0.05
7	2.04	2.04	0.00
8	2.13	2.21	+0.08
9	1.92	1.92	0.00
10	1.81	1.87	+0.06
11	2.00	2.00	0.00
12	1.92	1.81	−0.11
13	2.06	2.00	+0.06
14	1.62	1.62	0.00
15	1.72	1.70	−0.02
16	1.41	1.30	−0.11
17	1.22	1.22	0.00
18	0.93	1.02	+0.09
19	1.15	1.20	+0.05

Response to addition of cytochrome oxidase substrates

Paraphenylenediamine or hydroquinone was added in the presence of excess cytochrome C to breis prepared from different stages. Figure 2 summarizes the data obtained. The respiration level following the addition of substrate is, under the conditions of these experiments, proportional to the cytochrome oxidase activity. It is clear from the results that this activity is, as far as can be determined, constant throughout development. Consequently neither the rise in the respiratory rates of the intact embryos nor the fall in the respiratory rates of the breis observed in the later stages can be ascribed to variations in the content of this enzyme. The data obtained with added substrates are consistent with the view that the burst of respiration following cytolysis is due to the breakdown of controls, exerted by geometrical constraint, on existent substrates and enzymes.

It is worthy of note that the addition of the cytochrome oxidase substrates raises the respiration at all stages considerably above that attained by the brei alone. This might indicate that even in the early stages all the available active centers of the enzyme are not completely saturated by endogenous substrate when a brei is prepared.

The results of adding the cytochrome oxidase substrates with or without excess cytochrome C to intact eggs at different developmental stages are summarized in

Table II. For purposes of comparison the same table includes data on the rates of breis following similar additions. For the most part these breis were prepared from the same groups from which individuals were used for the intact embryo experiments.

It is clear that in comparison with the marked increases obtained with the breis, the addition of substrate has relatively little effect on the respiration of intact embryos. No consistent strong increase is obtained at any time from stage 3 up to and including stage 19.

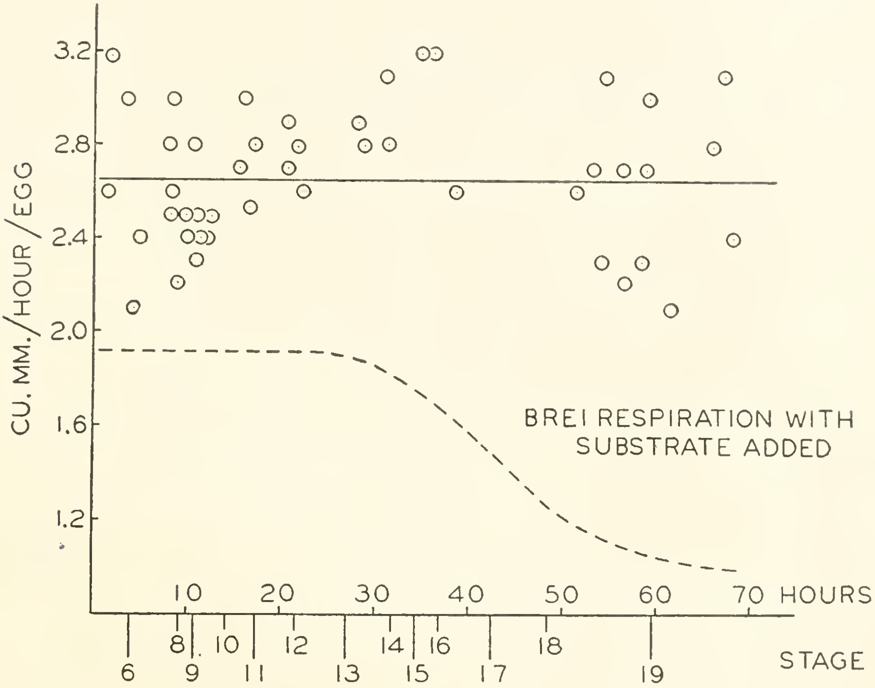

FIGURE 2. The effect on respiration rate of adding substrates of cytochrome oxidase to brei during development. The dotted line represents the curve taken from Figure 1. Single points represent individual experiments.

It is impossible to explain these results on an inability of these substances to penetrate the cells since all of the agents are effective in stopping further development and cause a severe bleaching of the melanin pigment. Both of these facts indicate with some certainty that substrates do penetrate the cells. Thus a simple lack of substrate does not limit the rate of respiration of the intact cells. Carrier immobilization would appear to be the most plausible explanation, although it is conceivable that endogenous substrate somehow forms a protective shell around active centers.

EXPERIMENTS WITH INHIBITORS

Assuming that the respiration is largely mediated through the cytochrome oxidase system, it was of some interest to examine the action of inhibitors of cytochrome oxidase. According to Keilin and Hartree (1939) both cyanide and azide are effective against this enzyme.

Response of brei and intact embryos to NaCN

In these experiments 0.001 M NaCN was used since it was found by preliminary experiments that higher concentrations did not give significantly greater depressions. The usual precautions suggested by Krebs (1935) were observed and appropriate mixtures of alkali and cyanide were used in the center wells. The results

TABLE II

Respiration (cu. mm./egg/hour) of brei and whole eggs on addition of cytochrome oxidase substrate. Substrate concentration M/50; cytochrome C 10^{-4} mM.

Stage	Whole egg	Brei	Whole egg substrate	Brei substrate	Change on adding substrate	
					Whole egg	Brei
3	0.21	1.6	0.20	3.2	−0.01	+1.6
	—	1.7	—	2.6	—	+0.9
	—	2.1	—	3.0	—	+0.9
4	0.21	—	0.20	—	−0.01	—
	0.20	—	0.20	—	0.00	—
	0.25	—	0.21	—	−0.04	—
5	0.17	1.8	0.26	2.1	+0.09	+0.3
	0.15	—	0.16	—	+0.01	—
	—	1.8	—	2.4	—	+0.4
7	—	2.2	—	2.2	—	0.0
	0.20	2.2	0.40	3.0	+0.20	+0.8
	0.15	2.2	0.20	2.5	+0.05	+0.3
	—	2.2	—	2.6	—	+0.4
	0.23	—	0.24	—	+0.01	—
	0.26	1.9	0.21	3.0	−0.05	+1.1
8	—	1.7	—	2.4	—	+0.7
	0.27	2.3	0.29	2.3	+0.02	0.0
	0.30	2.4	0.31	2.4	+0.01	0.0
8^{3+}	0.18	1.7	0.26	2.8	+0.08	+1.1
	0.20	2.1	0.22	2.4	+0.02	+0.3
	0.20	—	0.23	—	+0.03	—
8^{8+}	—	1.7	—	2.5	—	+0.8
8^{9+}	—	2.3	—	2.5	—	+0.2
8^{12+}	0.35	—	0.32	—	−0.03	—
	—	1.9	—	2.5	—	+0.6
10	0.40	2.1	0.60	3.0	+0.20	+0.9
	0.34	2.2	0.30	2.8	−0.06	+0.6
	—	2.0	—	2.7	—	+0.7

TABLE II—*Continued*

Stage	Whole egg	Brei	Whole egg substrate	Brei substrate	Change on adding substrate	
					Whole egg	Brei
12	0.45	1.9	0.50	2.6	+0.05	+0.7
	0.58	1.7	0.58	2.8	0.00	+1.1
	—	1.8	—	2.7	—	+0.9
	0.50	2.2	0.53	2.9	+0.03	+0.7
12^{8+}	0.57	—	0.70	—	+0.13	—
	0.60	—	0.56	—	+0.06	—
13	0.67	2.4	0.67	3.1	0.00	+0.7
	0.64	2.6	0.64	2.9	0.00	+0.3
	0.65	2.9	0.65	2.8	0.00	−0.1
13^{4+}	0.78	—	0.78	—	0.00	—
	0.75	—	0.63	—	−0.12	—
14	0.96	1.3	0.87	2.8	−0.09	+1.5
	—	1.6	—	3.1	—	+1.5
16^{1+}	1.1	1.3	0.83	3.2	−0.18	+1.9
	—	1.2	—	3.2	—	+2.0
	1.2	—	0.94	—	−0.26	—
16^{3+}	—	1.3	—	2.5	—	+1.2
18^{1+}	—	0.78	—	2.2	—	+2.1
18^{2+}	1.7	1.4	1.8	2.6	+0.1	+1.2
18^{3+}	2.1	1.0	2.2	3.1	+0.1	+2.1
	2.1	0.90	2.6	2.9	+0.5	+2.0
18^{5+}	1.6	0.98	1.2	2.3	−0.4	+1.3
	—	0.89	—	2.7	—	+1.8
18^{8+}	—	0.89	—	2.7	—	+1.4
18^{12+}	1.9	0.97	1.6	2.1	−0.3	+1.1
	2.1	0.90	1.8	2.7	−0.3	+1.8
	—	1.00	—	2.3	—	+1.3
19	2.4	1.0	2.8	2.4	+0.4	+1.4
	2.6	0.97	2.0	2.8	−0.6	+1.83
	—	0.85	—	3.1	—	+2.25

obtained are summarized in Figure 3. The data show that a strong inhibition of whole egg respiration is obtained at all stages. The respiration of the brei, while not diminished to levels quite as low, is also strongly depressed. Figure 4 gives the trend of cyanide sensitivity in terms of per cent inhibition and is based on calculations of average values from Figures 1 and 3. The fall in sensitivity of the brei in

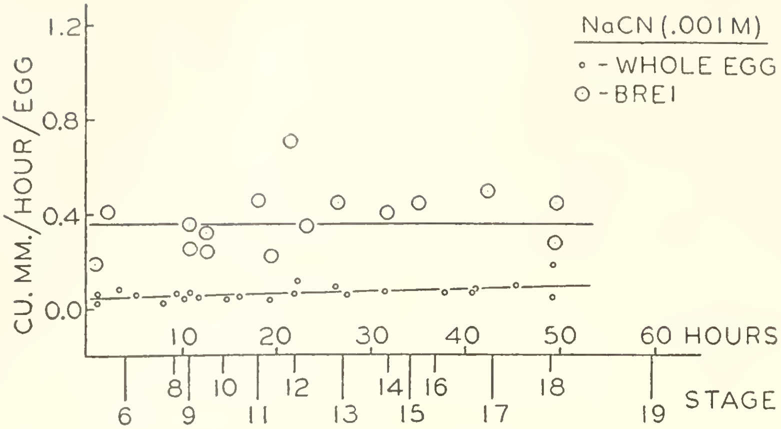

FIGURE 3. Respiratory rate in the presence of cyanide of breis and intact embryos during development.

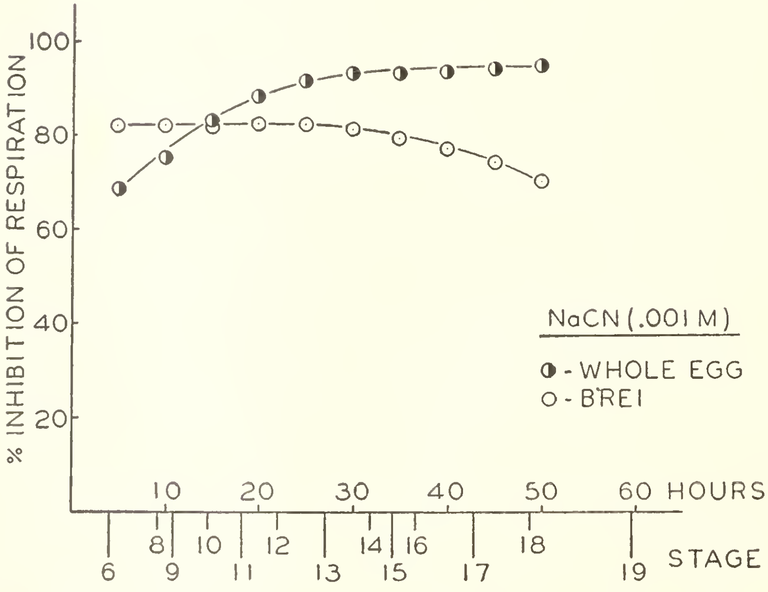

FIGURE 4. Cyanide sensitivity of intact embryo and brei respiration during development.

the later stages is a numerical consequence of the diminishing normal rate during this period which has been attributed to substrate limitations.

These data show that a major portion of the respiration of both brei and intact eggs is mediated via a cyanide sensitive system and that the increments in respiratory rate observed with development are due to the gradual augmentation in the activity of this cyanide sensitive system.

Response of intact embryos and brei to sodium azide

The same type of experiments as were carried out with NaCN were also performed with sodium azide at pH 6.7. The concentration selected was 0.005 M since higher concentrations up to and including 0.1 M did not result in more pronounced effect. The respiratory rates of intact embryos and corresponding breis obtained in the presence of azide are given in Figure 5. The percentage inhibitions at the various developmental stages calculated on the basis of averages from Figures 1 and 5 are shown in Figure 6.

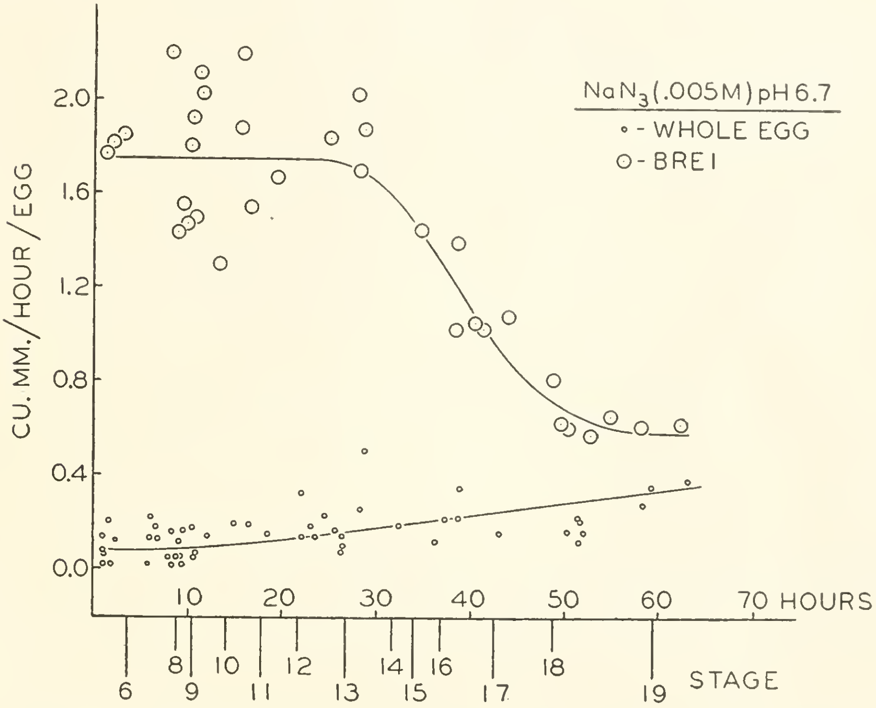

FIGURE 5. Respiratory rates in the presence of azide of breis and intact embryos during development.

In general (Figs. 5 and 6) the behavior of intact embryos towards azide is not very different from that with cyanide. However the residual azide insensitive respiration is consistently greater than its cyanide sensitive counterpart throughout development. Furthermore, unlike the cyanide insensitive respiration which remains constant, the absolute value of the azide insensitive respiration rises during development.

The actions of cyanide and azide on brei are strikingly different. Up until stage 13, 0.005 M azide can decrease the respiration of brei by only 10 per cent. It should be emphasized that this relative ineffectiveness of azide as a respiratory inhibitor against brei is not confined to this concentration only. As may be seen from column 2 of Table III it is true for concentrations as high as 0.1 M.

While Keilin and Hartree (1939) came to the conclusion that azide was virtually equivalent to cyanide in inhibiting the cytochrome oxidase system, recent experiments (Winzler, 1943; Stannard, 1939; Armstrong and Fischer, 1940) show that this conclusion is not universally applicable.

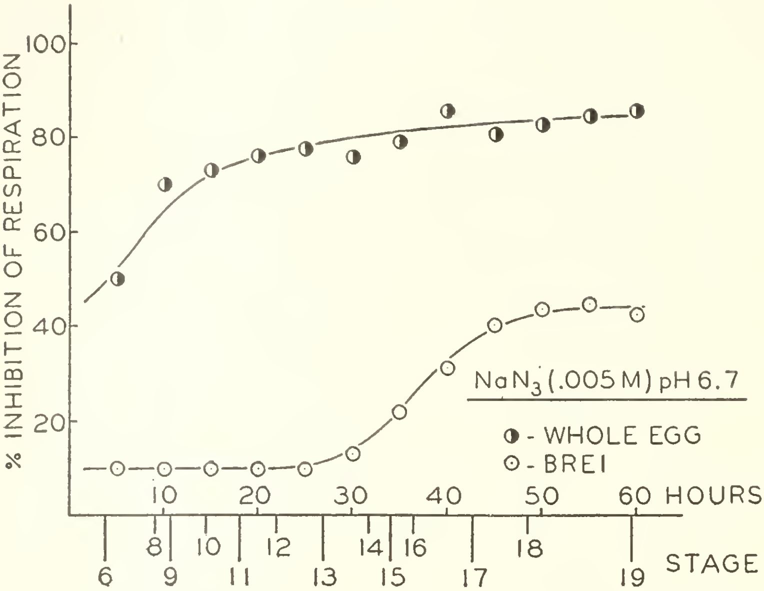

FIGURE 6. Azide sensitivity of intact embryo and brei respiration during development.

To test whether azide attacks the cytochrome oxidase in the breis in the concentrations employed as well as at higher ones, breis were prepared from embryos in stage 9 and placed in the main compartment of Warburg vessels with adequate amounts of cytochrome C and various concentrations of NaN_3. Sufficient paraphenylenediamine was placed in the side arms to yield a final concentration of 1/50th molar. After an adequate number of readings were taken to establish the rate without substrate, the substrate was introduced and the resulting respiration followed.

TABLE III

Effect of various concentrations of NaN_3 on O_2 consumption of brei when paraphenylenediamine is added. Rate is cu. mm./egg/hour. All eggs in stage 9.

M conc. of azide	Rate before substrate addition	Rate after substrate addition	Δ
0.000	1.91	2.51	+0.60
0.005	1.70	2.29	+0.59
0.01	1.80	2.50	+0.70
0.05	1.63	1.75	+0.13
0.10	1.65	1.67	+0.02

The results are given in Table III. Concentrations up to and including 0.01 M are unable to prevent the rise which occurs on adding substrate. The two higher concentrations tested, 0.05 and 0.1 molar, although ineffective against the respiration of the endogenous substrate, were capable of preventing the burst which normally follows the addition of paraphenylenediamine.

Similar experiments were carried out with cyanide (Table IV). Cyanide not only depresses the brei respiration to levels previously noted but in addition prevents any rise when the paraphenylenediamine is added.

TABLE IV

Effect of various concentrations of NaCN on O_2 consumption of brei when paraphenylenediamine is added. Rate is cu./mm./egg/hour. All eggs stage 9.

M conc. of NaCN	Substrate rate before addition	Rate after substrate addition	Δ
0.000	2.14	2.86	+0.72
0.001	0.51	0.51	0.00
0.002	0.32	0.38	+0.06
0.003	0.24	0.24	0.00
0.005	0.44	0.54	+0.10

These experiments with cyanide establish with some degree of certainty that the normal brei respiration is mediated by the Warburg-Keilin system. At least three important criteria for this assertion are satisfied: (1) The system exists in the brei. (2) The response to cytochrome oxidase substrate is cyanide sensitive. (3) The respiration of endogenous substrate is also cyanide sensitive. It is therefore necessary to assume that insensitivity of the brei respiration to azide is due to the latter's inability to combine with the cytochrome oxidase in the brei. It is conceivable that azide cannot combine readily with the cytochrome oxidase when the other enzymatic components and substrates of the cell are present in the extract.

It is certainly reasonable to expect that the sensitivity of a given enzyme system to a particular inhibitor will depend on the kind and amount of the substrate with which it is competing for active centers. It is therefore not surprising that cytochrome oxidase should be more sensitive to azide when in comparative isolation. That some such competitive mechanism is working may be seen from an analysis of the data in Table III and in particular from the fact that the two highest concentrations employed, while leaving the respiration of endogenous substrate relatively untouched, effectively suppress the increased oxidation when paraphenylenediamine is added. These findings would be expected if at the higher concentrations of azide the inhibitor could compete successfully for an active site on the enzyme surface with a molecule of paraphenylenediamine but not with a molecule of endogenous substrate. Further, it will be recalled (see Fig. 6) that azide sensitivity of the brei respiration starts to rise beyond stage 13 when the normal rate starts to fall due to substrate limitations. This finding would also be expected, since with substrate concentrate falling the ratio of azide to endogenous substrate rises and the azide can then start to compete successfully for active centers.

While the above interpretation fits all the data obtained on the relative azide insensitivity of brei it leaves unexplained the effective inhibitory action on intact embryos shown in Figure 5. If we interpret this inhibition as a reaction between the Warburg-Keilin system and azide we would necessarily conclude that this agent can combine with cytochrome oxidase in intact eggs but not in cell extracts. A plausible reason for this difference may be seen by focussing attention on substrate availability in the two situations. Here again we probably have a situation analogous to the increasing azide sensitivity of brei when the rate is falling i.e., less severe competition by endogenous substrate molecules for active centers as their concentration decreases. It is undoubtedly true that in the intact eggs the number of substrate molecules getting to the enzyme surface is severely regulated, permitting the azide molecules to compete successfully.

It is possible to explain the different responses of brei and whole egg to azide by assuming that in neither case can the azide combine with cytochrome oxidase in the concentrations employed. The capacity of azide to inhibit synthetic processes has been demonstrated for certain systems (Winzler et al., 1944; Moog and Spiegelman, 1942). Most important for the present discussion is the finding by Spiegelman and Moog (1945) that azide completely inhibits amphibian development at all stages including those between fertilization and gastrulation. From this point of view then, azide may depress the respiratory rates of intact eggs indirectly because it inhibits synthetic activities leading to substrate availability for enzyme action.

Discussion

The data presented find their most plausible interpretations in terms of structure orientation and the consequence of its destruction. The comparison of brei and intact embryos in the course of development as well as the determination of cytochrome oxidase content ruled heavily against enzyme or substrate synthesis as explanations for the rising respiratory rate during development. This emphasis on the internal geometry of enzyme and substrate and its variation as determining factors is a point of view that is becoming increasingly popular. Recent reviews by Korr (1939) and Commoner (1942) have stressed it as a criterion for evaluating data obtained from in vitro experiments. Both Runnstrom (1930) and Korr (1937) have discussed the sudden activation of the Warburg-Keilin system on fertilization of Arbacia eggs in terms of the relative positions of enzyme and substrate. Ballentine (1940a, b) who studied the dehydrogenase activity in the same material suggested similar considerations for these enzyme systems. It seems inescapable that more refined interpretations of physiological processes must consider not only what enzymes and substrates exist in the cell, but also where they exist. The present investigation which explains rising respiratory rate in terms of reorientation of existent components indicates that this is equally true for embryonic processes.

Summary

The respiratory rates of whole embryos and cell-free breis were determined at various stages of development. During early stages, when the respiration of whole

embryos is low, brei respiratory rate is high, the situation being reversed beyond about stage 17.

Cytochrome oxidase remains uniformly high throughout development as shown by the effects of adding substrate to breis. Externally applied cytochrome oxidase substrates have little effect on intact embryos although the agents are shown to penetrate. Cytochrome C is shown not to be a limiting factor in brei respiration.

Cyanide depresses the respiration of both breis and embryos. Azide has little effect on respiration of breis but is very effective in depressing oxygen consumption and development of intact embryos.

The results are discussed in terms of spatial orientation of enzymes and substrates.

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PINNOTHERES OSTREUM, PARASITIC ON THE AMERICAN OYSTER, OSTREA (GRYPHAEA) VIRGINICA[1]

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Introduction

The oyster crab, *Pinnotheres ostreum,* was first described by Say in 1817. It is an organism known for many years to oystermen and biologists alike although its habits and life history are but imperfectly known. In her monograph on the grapsoid crabs Rathbun in 1917 defined the adult characters of the genus and compared the numerous species which had been collected from many parts of the world in association with various mollusca, tunicates, annelids and sea urchins. This association has regularly been considered commensalism by most investigators. In the case of *P. ostreum,* however, the relationship is not clear for in 1892, Bashford Dean wrote that the crab was evidently annoying to the oyster for the palps sometimes show thickened out-growths or are malformed and stunted in size. Orton, in 1921, presented clear evidence in the case of the pea crab, *P. pisum,* from the mantle cavity of the mussel, *Mytilus edulis,* that the activity of the crab was definitely parasitic in nature. Ryder, on the other hand, supposed that the crab was of value to the oyster since the latter was said to feed on the clusters of bell-animalcules (*Zoothamnium* sp.) which are attached to the crab's shell. A consideration of the crab's stomach contents, however, showed that its food consists in great part of the minute organisms sought by the host in addition to small crustaceans not normally the oyster's prey.

A sudden increase in the numbers of *P. ostreum* in Delaware Bay in 1941 associated with definite erosions of the gills of the oyster and producing, or at least contributing to, the death of many oysters stimulated a re-examination of the crab. The data obtained (abstracted recently, Stauber, 1942) are the subject of this paper.

Life History

Atkins (1926) has described the post-planktonic stages in the developmental cycle of *P. pisum* from *Mytilus edulis* in British waters. The situation with *P. ostreum* is so similar that, except for morphological details, it seems to be the same. The invasive stages of the oyster crab are small, hard, flat crabs with a carapace 1.4–3.4 mm. wide (Figs. 1, 3, and 23). The sexes are separate and indistinguishable except for the number of pleopods and the genital apertures. Apparently copulation takes place after invasion of the oyster and perhaps is necessary, as Atkins suggests, in order for further development of the female crab to take place. In *P. pisum* she found that in all other stages of the female crabs the spermathecae contained some spermatozoa. Occasionally only one spermatheca was full but more often both were distended with sperms. Except for a few males similar in external appearance to the second stage females no other stage of the male *P. ostreum* is known and the male is believed to be free-swimming like the invasive first stage female.

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[1] The author wishes to express his deep appreciation for the many ways in which Dr. Thurlow C. Nelson aided and inspired this work.

[2] Now situated in the Department of Zoology, Rutgers University.

The second stage female differs markedly from the first stage being rounder in form, grey in color with prominent chromatophores, with a softer carapace and slenderer legs. In size, however, it measures only slightly larger (0.9–3.1 mm.).

The third stage female is still larger (2.6–4.4 mm. wide) but aside from this is quite like the second stage in color, shape and appearance. Higher development of the pleopods differentiates it from the latter.

The fourth stage female is pale yellow in color and is again larger (3.6–8.9 mm. wide).

The adult female, or fifth stage crab, ranges in size from 6–14.9 mm. and is the form of *P. ostreum* commonly known. Indeed, except for this stage, only a few males and none of the other female stages have ever been recorded. Several moults of the fifth stage female crabs are possible and there is some overlapping of sizes of the crabs in various stages due to time of invasion, food supply, size of the oyster invaded, etc. No data are available on the rate of growth or time lapse involved between the various female stages. Only moults of fifth stage females and one moult of a third stage female into the fourth stage have been obtained in the laboratory. In one dredged oyster a fifth stage female and a moulted shell were obtained within the same bivalve.

Ovigerous females are seen in the summer and autumn of the year and Atkins believes that *P. pisum* may become adult in a single year. There is reason to believe that such is the case with *P. ostreum* also. Judging by the size of the mature female crab, with reference to the male copulatory pleopods, it seems unlikely that a second copulation takes place. Therefore, if the large crabs live more than one year, which is quite probable, they must have received sufficient sperm in the one copulation to last for several batches of eggs or become capable of producing only infertile eggs. Orton and also Atkins found the majority of large females of *P. pisum* to have full spermathecae but an occasional large female with empty spermathecae led the latter to postulate that copulation may occur more than once.

After a time, the length of which is still unknown, the developing embryos hatch from the eggs attached to the pleopods of the mother producing a zooea which has been described for *P. ostreum* by Birge (1882) and again by Hyman (1924). In our own experience an ovigerous female was obtained from an oyster dredged as late as October 19, 1942. The embryos then were almost ready to hatch and showed a pulsating heart, a fully formed abdomen, eyes, a digestive gland, and appendages. Hatching began four days later and large numbers of the first zooea were available for study.

Subsequent planktonic stages of this crab are still unknown but judging by Lebour's (1928) criteria (the relatively primitive condition of the first zooea) three or more zooeal stages are probably present and possibly also a megalopa. Eventually, however, the free-swimming invasive male and female crabs are formed completing the cycle.

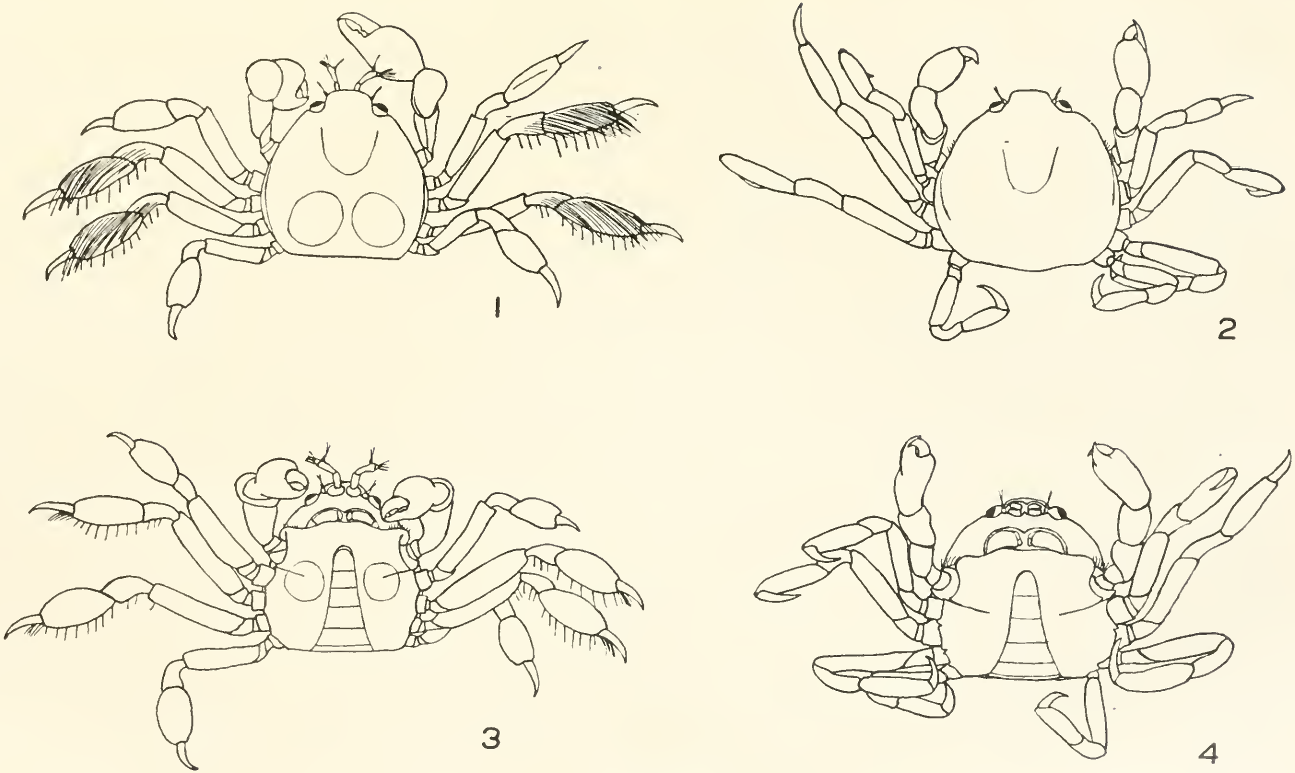

PLATE I

First and second stage female *Pinnotheres ostreum*. × 10

FIGURE 1. Dorsal view of Stage I female. Hard

FIGURE 2. Dorsal view of Stage II female.

FIGURE 3. Ventral view of Stage I female.

FIGURE 4. Ventral view of Stage II female.

Morphology of the Various Stages of the Crabs

The male

In contrast to *P. pisum,* the male *P. ostreum* has been considered an unusual find, Rathbun (1917) reporting only two specimens in the National Museum collection. In size it is much more restricted than the male *P. pisum,* the carapace usually being from 1.5–3.4 mm. wide with sizes below 1.6 and above 2.5 mm. being rarely seen. The mean carapace width of 144 specimens was 2.13 ± 0.19 mm. Furthermore, unlike all but the first stage female from which it is almost undistinguishable, its shell is well fortified with calcium salts probably making it as hard for its size as species of free-living crabs. In color it is dark brown with two large distinct almost circular pale white spots, visible both on carapace and on the sternum. On the posterior half of the dorsal side they are situated in the branchial regions. On the ventral side they flank the groove which contains the abdomen and are situated at the level of and mediad to the first pair of pereiopods or walking legs. In a male, whose carapace was 2.0 mm. wide the dorsal spots were roughly 0.6 by 0.5 mm. On the ventral side of the same crab the pale areas were only 0.45 mm. in diameter. The carapace is flat dorsally, sub-circular in shape with an advanced, more truncate front than is present in the later females. The posterior margin is straight and the widest part of the carapace about $\frac{4}{7}$ the distance from the anterior end. The eyes are well developed. The front is about $\frac{2}{7}$ as wide as the carapace. The lateral margin of the carapace is thin and rather sharply bent from the dorsal side. Antennules are large and antennae small. The epistome is well defined. The buccal cavity is crescentic, arched and very broad from side to side but very short fore and aft. The external maxillipeds completely close the cavity and consist chiefly of the merus fused with the ischium and with the flagellum attached to the inner end. Carpus of the first segment of the palp or outer maxilliped, short, oblong; propodus more elongate, rounded; dactylus inserted behind middle of the propodus, minute and slender. Chelipeds (Fig. 5) [3] are stout, merus and carpus not slender as in the later females. Propodus slightly flattened inside, swollen outside and strongly widened from proximal toward distal end, narrowing again so that width at base of dactyli is considerably less than the greatest width of propodus. Both margins of propodus are convex. Fingers are stout, especially the immovable one with the tips hooked past each other when closed. The movable finger has a small tooth which fits between two protuberances on the immovable finger. Stiff hairs project from the gripping surfaces of both fingers.

The pereiopods are markedly flattened with the propodi almost spatulate, the third leg being slightly longer and stouter (Figs. 6–9). The propodi are widest about centrally, being half as wide as long and over twice as wide as thick (0.42 × 0.18 mm. in one case). The posterior border of the propodi of the walking legs is

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[3] Although Figures 5–9, inclusive, were drawn specifically from a first stage female crab, the respective appendages of the same stage male are indistinguishable.

Plate II

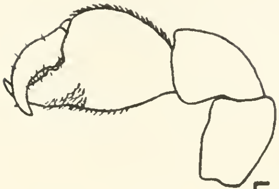
Ventral view of chelipeds and pereiopods of Stage I and II female *Pinnotheres ostreum.* × 54

Figure 5. Cheliped of Stage I female.

Figures 6–9. Pereiopods of Stage I female.

Figure 10. Cheliped of Stage II female.

Figures 11–14. Pereiopods of Stage II female.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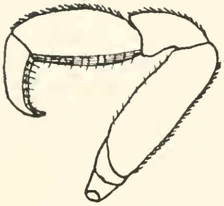
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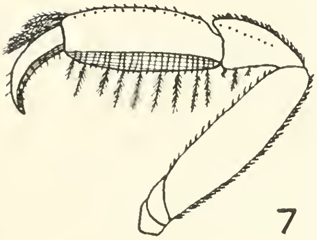
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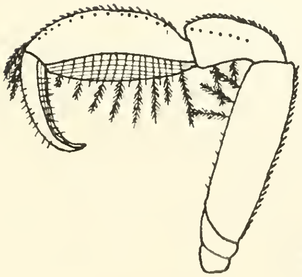
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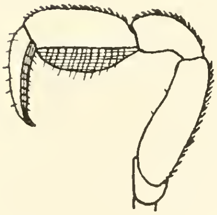
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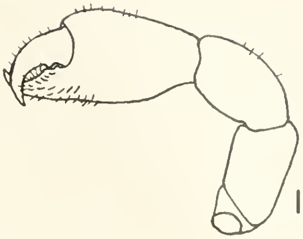
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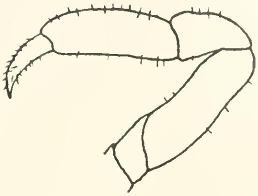
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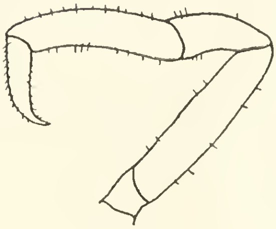
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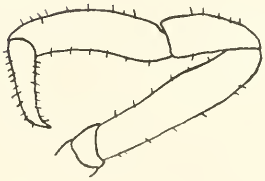
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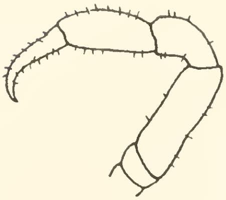
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Plate II

thickened. The amount of thickening is progressively greater from first to fourth walking leg, being not quite half the total width of the propodus of the fourth leg. The thickened portion seems to be constituted of the same material as the rest of the exoskeleton. Not only does it show longitudinal striations as though produced by successive depositions of chitin but the whole thickness is pierced by the hairs which protrude from the posterior border of each of the propodi. The dactyli of the same legs also show a similar thickening of the posterior which is likewise pierced by the protruding hairs. Unlike the female, the dactylus of the third leg of the male is longest but it is not markedly longer than that of the second which is almost as long and a trifle more curved at the tip. Also unlike the adult female, the dactyli of the second and third legs are only slightly more or less than half the length of the propodus of the same leg. There are two rows of long plumose hairs on the second and third legs. One row extends postero-dorsally from the anterior edge of the dorsal surface in a line running from midway on the carpus to the distal end of the propodus. Some of the hairs are over 0.6–0.9 mm. long. The other row extends backward from the posterior border of the flattened leg also extending from middle of the carpus to distal end of the propodus. These hairs are only about half as long as those on the anterior edge. The other joints of these legs and the other legs only possess short hairs or setae which are stout and plumose and are especially abundant on the anterior edges of the propodus, carpus and merus. We consider that these flattened, hairy legs are adaptations for free-living existence. Indeed, the first stage crabs are fair swimmers and reach the oysters by this means. The sides of the abdomen are almost straight and become narrower from the third to the seventh segment (Fig. 16). The terminal segment is arcuate. The abdomen fits neatly into the grooved sternum. A locking apparatus, somewhat like that described by Atkins for *P. pisum*, is present in the male *P. ostreum* (Figs. 15 and 16). It consists of a pair of almost conical chitinous knobs (on the fifth thoracic segment) along the side of the grooved area where the abdomen fits and a pair of larger blade-like protuberances on the sixth segment. Both sets of knobs project slightly anteriorly, ventrally, and medially.

Instead of fitting into grooves on the abdomen there are shelf-like projections on the fifth and sixth abdominal segments. The shelf on the fifth segment is much shallower and smaller than that on the sixth. The knobs on the thorax are hooked under these shelves making a close-fitting efficient locking mechanism that can easily be undone by the crab through extension of the abdomen and lifting but which is

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PLATE III

Some details of structure of *Pinnotheres ostreum*

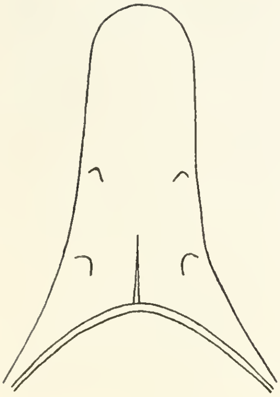
FIGURE 15. Ventral view of the sternum of a male crab (Stage I) showing the chitinous knobs of the locking mechanism. × 20.

FIGURE 16. The abdomen of a Stage I male crab showing the copulatory pleopods. The first appendage on the right side was removed to better expose the smaller second appendage. Figure also shows the shelves of the locking mechanism. × 20.

FIGURES 17 AND 18. First and second pleopods, respectively, from the left side of a Stage II female crab. × 166.

FIGURES 19 AND 20. First and second pleopods, respectively, from the left side of a Stage III female crab. × 43.

FIGURES 21 AND 22. First and second pleopods, respectively, from the left side of a Stage IV female crab. × 22.



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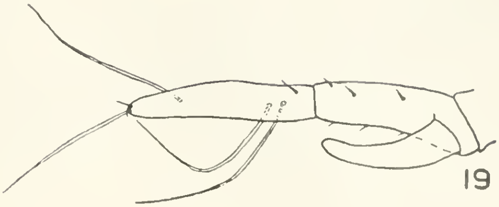

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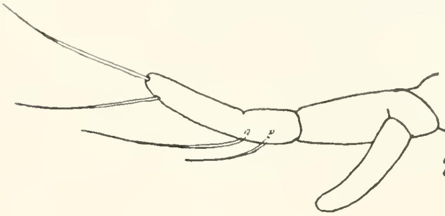

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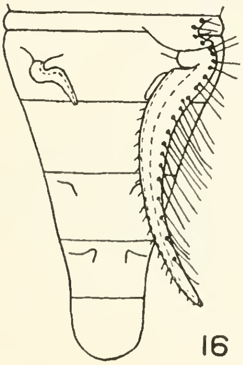

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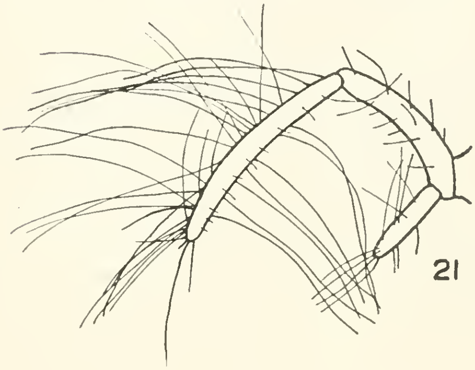

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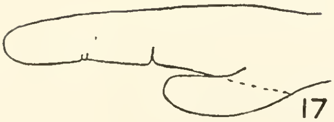

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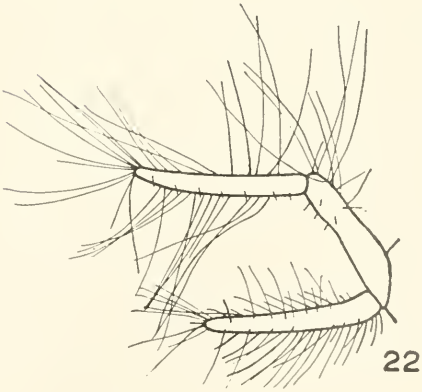

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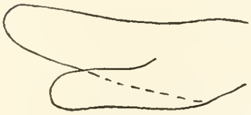

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Plate III

difficult to dislodge in the living animal where the stronger the flexing strain the more tightly the knobs lock.

The only thoracic somite dividing suture on the sternum is seen extending mediad from behind the coxa of the cheliped to a point a little more than midway to the groove where the abdomen lies. This is between the fourth and fifth thoracic segments. The copulatory organs of the male are large, like those in *P. pisum,* the first is blade-like and hairy and the second is rod-like (Fig. 16) and almost hairless. The tube runs along the medial side of the first appendage and the second appendage fits into a groove running across the under surface of the first appendage near the base of the terminal segment. The first appendage is more slender and scimitar-like than the same appendage on *P. pisum.*

First stage female

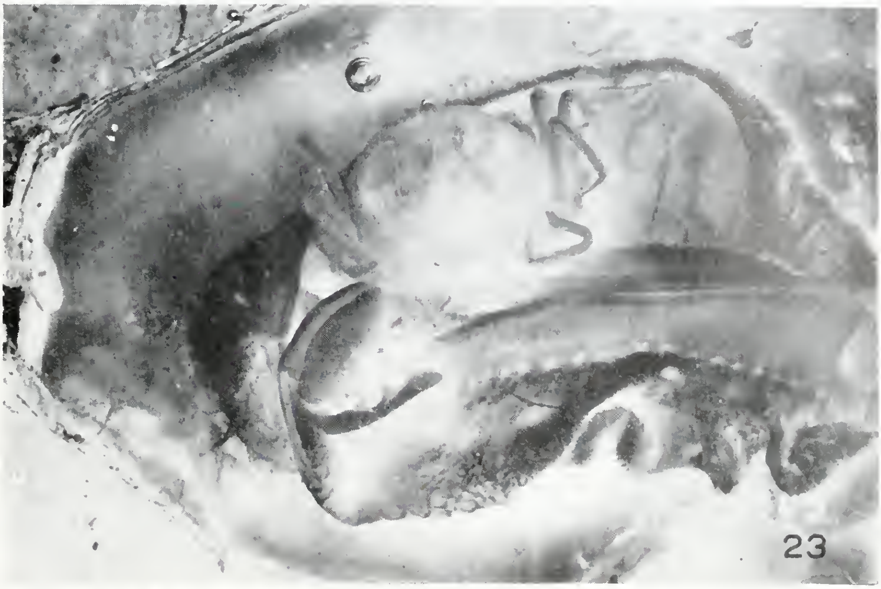
This is the female stage which invades the oyster (Figs. 1 and 3). It is superficially indistinguishable from the male although it is usually slightly smaller on the average (1.4–2.4 mm.). The mean of 187 measurements of width was 1.83 ± 0.14 mm. Not until careful examination is made of the abdomen and the sternal groove into which it fits can differentiation be made.

For example, there is only one pair of chitinous knobs (corresponding to the anterior pair of the male) and consequently only the terminal or corresponding pair of shelves on the abdomen (on the sixth segment). The genital aperture is small and located just posterior and medial to the base of the chitinous knob on each side. The relationship is thus almost precisely like that in *P. pisum.* The abdomen in the female has slightly straighter sides with the terminal segment having a lower arch. It lacks one pair of locking shelves and the segmentation between 3 and 4 and between 4 and 5 is much less distinct than in the male. Furthermore, the first segment is widest unlike the male where the third segment is widest. Such differences are very minor and usually cannot be determined *in situ* but only by removing the abdomen for comparison. Then, however, these differences are quite constant. Finally, the abdomen of the female bears four pairs of minute appendages, the first one of which is biramous, the second has a bilobed tip, presaging its eventual biramous state, and the other two are simple knobs. The walking legs are like those of the male.

Second stage female

This stage hardly exceeds in size the range of the first stage female. The carapace width of 67 specimens ranged from 0.9 to 3.1 mm. (mean 2.1 ± 0.23 mm.). The moult which occurs involves more differentiation than growth. The carapace is now smooth, shining, thin and membranaceous and, unlike the previous stage, yielding to the touch. It is convex from before backward and the lateral margins are thick and bluntly rounded. The front is still about 2/7 the width of the carapace but now it is less truncate and does not project quite so far forward (Figs. 2 and 4).

The chelae are slightly more slender with the propodus widest more distally, nearly at the base of the fingers (Fig. 10). The pereiopods (Figs. 11–14) are slender and subcylindrical with delicate hairs on the last three segments. No such plumose hairs are present as described on the Stage I crabs. Propodus is not flattened on pereiopods as in Stage I nor does it broaden out very much, being only



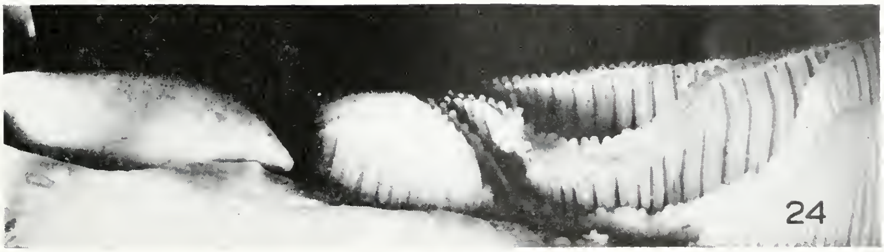


PLATE IV

Photomicrographs of *Pinnotheres ostreum* and gill-eroded oysters. I wish to express my appreciation to Dr. T. C. Nelson for these. He not only took the photographs but found the particular specimens displayed. All × 3.

FIGURE 23. View of large-crab type gill erosions in an oyster. Note the presence of both a large Stage V female crab and a small Stage I male crab. The demibranchs of the oyster are shorter than normal and the ventral mantle cavity much enlarged.

FIGURE 24. Small-crab type erosions of gills. Note the sharply-delimited, punched-out area involving three demibranchs.

slightly wider than the merus of the same leg. The third leg again is largest and the dactyli of second and third legs nearly equal in length.

The abdomen is approximately the same shape as in the first stage. The ratio of carapace width to abdomen width is 2.46 and of abdomen length to abdomen width is 1.36. The first is still the widest segment. The terminal segment is only slightly wider (1½ times) than long in this stage. The sternum is still deeply grooved to hold the abdomen flush. Chitinous knobs are still to be seen on the thorax but the mechanism is now weakened by the delicate nature of exoskeleton.

This is obvious the first time one attempts to lift the abdomen. In Stage I males and females this is accomplished with difficulty and often the abdomen is injured in the process. In this and subsequent stages lifting of the abdomen is easy. Here is an adaptation to the sheltered life within the mantle cavity of the oyster which free-living first stages do not show. The legs also, no longer needed for swimming, have become slender and relatively smaller with each stage of development.

The abdominal appendages are but little advanced over those seen in the first stage female though in some instances they seem farther along than others. This and subsequent stages, while rather clearly defined, do vary quite broadly within the stage. In some there seems to be little change over the appendages of the first stage female, in others the biramous character of the first two pairs is more clearly marked. The appendages are still hairless, small and relatively undifferentiated (Figs. 17 and 18). There are many chromatophores scattered over carapace, sternum and abdomen. The legs have few of these black chromatophores although the chelae are well supplied. Consequently, the crab in this stage, against a light background, appears mottled grey with translucent appendages. Against a dark background, the appendages show up better and appear opaque. The later stages, especially the third stage female, are quite similar in color but cream-colored specimens are seen in increasing abundance. This is due chiefly to the decrease in numbers of these chromatophores and dispersion of pigment within them, and an increasing opacity of the organs within the crab. The adult female is always cream-colored except for the reddish outline of the mature gonad, the anterior parts of which are seen through the carapace from above and the posterior parts through the abdomen from below.

Third stage female

The chief differences are those of size, shape, and relative size of abdomen and character of the abdominal appendages. The width is from 2.6–4.4 mm. (mean of 31 specimens was 3.17 ± 0.40 mm.). It was found less frequently than first or second stage crabs in 1941 but has been relatively more abundant in 1942. The second and third walking legs are nearly equal in length with the dactyli also almost the same length. The average ratio of carapace width to abdomen width in eleven specimens was 2.0 and that of abdomen length to abdomen width was approximately 1.1. Thus the width of the abdomen has relatively increased slightly over both the length of the abdomen and the width of the carapace. In most of the specimens the third or fourth segment was widest. The terminal segment of the abdomen now has a flatter arch, usually being over twice as wide as long. The abdominal appendages, especially the first two pairs, are clearly segmented and possess a few distinctly plumose hairs (Figs. 19 and 20). The edges of the abdomen now extend beyond the broadened and shallower depression in the sternum which in earlier stages wholly contained the abdomen. There are hairs on the lateral edge of the abdomen in this stage. These hairs are more abundant on the more proximal segments and are quite variable in number. They are stout, plumose and rarely exceed 0.15 mm. in length.

Fourth stage female

Specimens on hand range in size from 3.6–8.9 mm. The abdomen is much broader now and just reaches the coxa of the legs in the majority of specimens.

It extends forward almost to the posterior edge of the mouth. The sternal depression is much broader in this stage though the abdomen overlaps it considerably. The third or fourth abdominal segment is widest and the abdomen wider than long even when pressed out flat. The carapace-abdomen width ratio has also further decreased and is now nearly equal to 1.0. The terminal abdominal segment has broadened to such an extent that it is now three to five times as wide as it is long. The abdominal legs are almost fully developed and well supplied with hairs (Figs. 21 and 22). The hairs along the lateral edge of the abdomen are more numerous and large and are present on all segments as a uniform fringe in the fourth stage crab.

The orbit has hardly increased in size and the diameter of the eye is practically the same. Thus the relative prominence of the eyes has decreased markedly. The carapace is now much more convex so that the front appears to be ventral in position. The abdomen is very convex externally and cannot be flattened without tearing the exoskeleton. Ventrally the hollowed abdomen is suited for carrying the masses of ova.

Fifth stage female

This is the stage commonly seen. The prominence of the reddish gonads identifies it in most cases though some fifth stage females are found with immature gonads. A few apparently fourth stage crabs show mature gonads, however. The features which differentiate the fourth and fifth stage females are rather slight. The abdomen is now wider than the carapace, extending to the basis of the walking legs in many cases. The terminal segment is more than five times as wide as long. The abdomen projects forward to a point anterior to the mouth which it covers except when feeding. For this act it rolls inward the terminal segments exposing the mouth parts as described for *P. pisum* by Orton. The size ranges from 6.0–14.9 mm. (34 specimens measured). It is possible that adult size is determined in part, as Atkins found for *P. pisum,* by the size of the host invaded. Even here, however, the range is great. Thus, a fifth stage crab 9.0 mm. wide was found in an oyster only 29 mm. long. Its abdomen was 12 mm. wide and extended beyond the coxopodites. No studies relating host size with crab size have been made but such a factor would account partly for the overlapping sizes of the various stages of the female crab. Another factor would be subsequent moulting after the fifth stage is reached. This is known to happen and several fifth stage females have moulted in the laboratory. Since only one moult of an earlier stage has been obtained which resulted in the production of a fourth stage female from a third stage crab, it is not known whether all earlier moults involve metamorphosis or whether growth moults can occur between stages. Biological variability and amount of available food are probably additional factors in size variations.

The walking legs of the adult female are different in their proportions from the earlier stages for the second leg is distinctly longer than the others and the dactylus of the second leg is more than two-thirds as long as the propodus. In our specimens, however, it does not seem to be regularly seven-eighths as long as Rathbun described it.

Two ovigerous females are present in our collection, the first measures 14.0 mm. across the carapace but the other only 8.9 mm. This is a further indication of the variability existing in the stages of the crab.

Second stage male

The presence of a number of specimens similar in external appearance to second or third stage females (body shape, exoskeleton yielding to the touch, slender, translucent legs, and grey or cream in color) has been noted. Thirteen measured specimens averaged 3.1 mm., ranging from 2.0–4.8 mm. Shape of abdomen and presence of typical male pleopods clearly identify these crabs as males. The pliable abdomen, however, only has one pair of the shelves of the locking mechanism though the sternum still shows both pairs of chitinous knobs. Like the females (except those of Stage I) the exoskeleton is delicate in these soft-bodied males and the abdomen can be easily lifted from the sternum even in living crabs. Since these crabs have been found in appreciable numbers it is quite possible that they may serve as partners in copulation with larger females of later stages though no evidence is available for or against this possibility. The size range would indicate that at least one, and probably two, moults can occur beyond the typical hard-shelled first stage males. It is also possible that these atypical males are the result of some sort of parasitism as Mercier and Poisson (1929) have reported for *P. pisum*. They found that males parasitized by the entoniscid, *Pinnotherion vermiforme,* resembled immature females in increased size and decreased firmness of the carapace and in the diminished number of hairs and greater slenderness of the legs. Parasitized female *P. pisum* were not perceptibly modified in these characteristics.

PRESENCE OF PINNOTHERES IN OYSTERS

All the stages of the crab described in the preceding pages were found in oysters from Delaware Bay in both 1941 and 1942. Although multiple infestations occurred involving various combinations of the crab stages, no case has been found containing more than a single specimen of either the third, fourth, or fifth stage crabs. First stage males and females were in great abundance in 1941 but were much less frequently seen in 1942 either per oyster opened or proportionate to the other crab stages. This is indicated in Table I where the same grounds of oysters were sampled in the two years. The only differences were that the oysters in each case were a year older in 1942 and that a twenty-five to thirty-three per cent mortality had occurred on each ground during the intervening winter.

TABLE I

Distribution of numbers and stages of P. ostreum *in oysters from two grounds sampled in consecutive years*

Ground	Year	Mean no. of crabs per oyster*	Max. no. of crabs per oyster	Oysters with crabs	Stage I crabs	Stage V crabs
A	1941	6.0	18	80%	91.4%	1.2%
	1942	1.1	3	73%	41.7%	33.3%
B	1941	4.2	19	80%	92.0%	1.6%
	1942	1.1	5	55%	50.0%	30.0%

* Based on total number of oysters in the sample.

We offer the data in this table also as additional evidence in favor of Atkins' belief (although she was working with *P. pisum*) that growth to maturity takes place within a year's time. The marked increase in both absolute and relative numbers of Stage V crabs in relation to a marked decrease in total crab population is more than suggestive.

The greatest number of crabs in a single live oyster was seen in 1941 when 262 crabs were found in an oyster measuring 85 × 46 mm. This oyster was in a sample dredged from Ground C already showing a high oyster mortality. Of the other live oysters in this sample, 54.6 per cent had more than 10 crabs present within the valves. Other high counts were 32, 63, 65, 81, 112, 134, 154, and 165 crabs per oyster. Multiple infestations have occurred even in small oysters, one dredged from Ground C, measuring only 32.4 × 14.7 mm., containing eighteen first stage crabs.

Besides oysters containing only a single specimen of any crab stage, almost all conceivable combinations of crabs have been found among the multiply-infested oysters except those involving the larger stages.

The only other cases of multiple infestations with crabs of the genus *Pinnotheres* were reported by Ohshima (1927). As many as seven male *P. latissimus* were found within the shells of a single specimen of *Paphia*.

The crabs when present in small numbers are usually found in the mantle cavity but in the heavier infestations crabs are found throughout the water conduction system of the oyster. An extreme case is that of the multiply-infested oyster dredged in September, 1942. The mature fifth stage female crab and five first stage crabs were found on the oyster's gills, three other first stage crabs were found in the cloacal chamber and one each in the promyal and the right and left suprabranchial chambers. In one interesting case the oyster when opened disclosed a Stage V female on the gills and a Stage II female in the promyal chamber. In another case three Stage I females were found one each in the promyal and left suprabranchial chambers and one at the exit of the promyal chamber.

When only a single crab is present it is usually found on the gills (in the mantle cavity) and females of Stages III, IV, and V have been found only in this area. We may state, therefore, that this is the normal position and that aberrant positions increase with the number of the invading crabs.

Orton (1921) reported a similar state of affairs with reference to *P. pisum* in *Mytilus edulis*. Observing the feeding of these crabs through windows in the mussel's shell he noted that the larger crabs sit on the middle of the mussel with a pair of gills on each side. The smaller crabs, he reported, may be found anywhere in the mussel but generally on the gill.

In Delaware Bay, oyster crabs are found in oysters from every area sampled to date. Since this included oysters well up the bay in regions of relatively less saline waters we may state that *Pinnotheres ostreum* is able to survive wherever the oyster can. Such a statement needs some qualification, however. Invasion of oysters by a new brood of young crabs probably occurs in late summer and autumn when the haligraphic picture of the bay waters (based on extreme data for more than seven years) is almost always at or above the mean annual level of salinities in the bay (Stauber, 1943). The meager data at hand indicate that if markedly low salinities prevailed at this critical time survival of the crabs would probably be greatly lessened. Crab-infested oysters subjected to adverse environmental conditions throw considerable light on this problem. Death of first stage crabs within the oyster has occurred as a result of over-winter storage of oysters in pits in the ground and in a home refrigerator and as a result of the subjection of oysters to periods of low salinities (spring of 1941 and late autumn of 1942 on the beds far up the bay). It is especially notable that the later stages of the female crabs were able to survive these same adverse conditions. The latter fact is clear evidence that the adaptation of the crab to life within the oyster, as is indicated by the morphological changes of the female crab, involves physiological changes as well. Since the adverse situations described above all involve persistent closure of the oyster the female crab acquires the ability (which the oyster is well known to possess) to survive periods when cessation of the food supply and interruption of a steady stream of oxygenated water for respiration occur.

Invasions of oysters by *Pinnotheres* most probably occur in the late summer and autumn. This statement is based on several types of observation. The few ovigerous females seen have been encountered only during this period. Besides, crab zooeae of various species are found during these seasons. Furthermore, the 1941 mortalities began to be noticed in September of that year. Finally, infestation of oyster spat only a few months old was observed several times. Such invasion could only have occurred in the late summer or early autumn.

The heavy crab infestations of oysters reported in Table I were extreme values. In most areas sampled fewer than twenty-five per cent of the oysters opened contained crabs of any size, Stage V females being in the great majority. In such samples multiple infestations were very unusual. This is as valid for 1941 when the general incidence was much higher as it is for 1942.

Oysters of all sizes have been found infested with crabs. All stages of crabs have been found present though no later stages of females have been seen in oysters less than one year old for reasons which are obvious. The smallest infested oyster was one less than four months old from the egg and only 20 × 16 mm. in size. A second stage female crab measuring only 0.9 mm. wide was found in the mantle cavity. The left mantle was perforated and the inner right demibranch slightly eroded. These observations indicate that growth and differentiation of the crab and perceptible damage to the oyster can occur within a relatively short period of time.

The smallest oyster yet opened which has contained a mature fifth stage female was an oyster probably less than two years old and measuring only 34 mm. long. The crab had a carapace 9 mm. wide and large-crab type gill erosions were present.

Mode of Feeding

Orton's description of the feeding of *P. pisum* in *Mytilus edulis* is remarkably similar to the method of feeding of *P. ostreum* in the oyster. The larger crabs are stationed with ventral side approximated toward the inner aspect of one mantle and facing away from the oyster's gills toward the ventral margin of the shell or into the incurrent stream of water. Usually two of the oyster demibranchs project over and above the carapace of the crab and not in regular contact with the crab. The other two demibranchs are close to and at times in contact with the posterior part of the ventral surface of the crab.

The oyster feeds by straining sediment and plankton (chiefly diatoms and dinoflagellates) from the water.[4] A portion of these strained particles mixed with mucus is passed anteriorly in trails along the marginal furrow of each demibranch. The palps of the oyster constitute a further sorting apparatus and some of the strained material is rejected by the palps. This rejected material is passed posteriorly and ventrally along a ciliated path on the inner aspect of the mantle to a point near the ventral edge of the shell from which it is ejected by the blowing force of the sudden closure of the oyster's valves. If the oyster crab confined its feeding activity to the constant stream of rejected material on its way from the palps it might be of some value to the oyster and could possibly be called a symbiont or at least a commensal. However, in our experience the rejected masses are too bulky to easily fit into the crab mouth and most of this material is eventually thrown off by the crab after attempting to break it up into suitable particles by the action of the mouth parts. By reaching beneath its abdomen with its claws and also by disengaging the tangled mucus-food masses caught in its legs, it obtains newly-formed, delicate, strings of food much more capable of being handled by the mouth parts and ingested. I have seen such strings completely and quickly engulfed. The crab often combs the other legs with the chelipeds to free them from entangled food masses. The mucous masses are then passed to the mouth where the mouth parts in turn gather and press the strings of food into the mouth. The fourth or last pair of walking legs are frequently worked up over the carapace of the crab to scrape forward within the easy reach of the chelipeds any food strings which may fall from the other demibranchs or even to disengage them from the marginal furrow. The large crabs range in the mantle cavity from the place where palps and gills meet to a point ventral to the adductor muscle. Under favorable conditions they are seen to be very active, almost constantly moving legs or chelipeds.

The process of feeding, however, is wasteful and much of the material brought to the mouth is handled several times before it is ingested with appreciable amounts thrown off by the mouth parts. In one case a large crab was straddling the stream of rejected mucus-food passing to the edge of the mantle from the palps yet in more than fifteen minutes' steady observation it failed even to attempt to pick it up. In the meantime the combings of the walking legs were being vigorously prosecuted.

Rathbun remarks that the food of the oyster crab is composed of the same organisms which constitute the food of the oyster plus small crustaceans not normally the food of the oyster. The complicated feeding mechanism of the oyster is chiefly one of straining particles from the water and then sorting out and discarding the larger of these particles. Much of the food passing along the marginal furrow is destined to be discarded because of particle size. It is not surprising, then, that small crustaceans (Rathbun) are included in the crab's diet in addition to the entangled smaller organisms eaten by the oyster.

Observations on the feeding of the smaller stages of *P. ostreum* (especially first and second stages) throw further light on the mechanism producing the gill damage to be described later. In maintaining its position on the demibranchs the pointed dactyli of the walking legs are regularly seen to pierce the gill tissue. In order to maintain its position several nearby plicae may be stretched out of position by the pull of the inserted dactylus. When the leg is moved the area pierced stands out as an opaque spot on the demibranch. It appears like a condensation of tissue either by compression of surrounding parts or by invasion of leucocytes. This area is visible to the unaided eye for several hours. Some of the walking legs have been observed to scrape the gill in an attempt to engage mucous strings of food moving on the filaments (especially the principal filaments in the folds between the plicae). This scraping action may be very vigorous and may be likened to the child's use of its fingers to clean the frosting from the egg beater. Occasionally the dactylus of the scraping leg catches in a portion of the gill (ostium). The crab strains and the gill tissue stretches. When the pull becomes hard enough the leg finally completes its motion (due to the local tearing of the gill) and the affected portion of the demibranch springs back into position. The leg and cheliped would then be approximated and the combing would proceed even when no food material had been obtained by the active leg. (Since many of these observations were made in very clear water there was little material to be filtered by the oyster.) Small crabs have also actually been seen to grasp the delicate gill tissue with the cheliped and put tension on it, pulling it out of normal alignment. When released the gill was seen to be definitely injured.

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[4] This description is based chiefly on the findings of Yonge (1926), Elsey (1935) and especially Nelson (1938 and personal communications).

In all stages of the crabs the activity was seen not to consist so much of movement from place to place but chiefly movements of legs and chelipeds related to the act of feeding.

Injury to the Oyster Gill and Gill Regeneration

Although no extensive erosions of gills were produced by crabs in the laboratory, nevertheless, the observations recorded and the activities and injuries discussed above are sufficient to account for the extensive gill damage regularly seen in crab-infested oysters.

Two general types of gill damage were noted associated with large and small crabs respectively. The large-crab type erosion (Fig. 23) shows extensive shortening in the height of one or more demibranchs over an area reaching from the anterior end of the gills where they meet the palps to a point usually ventral to the adductor muscle. The affected demibranchs may be even less than half as high as would be expected normally or as the unaffected demibranchs of the same oyster. The outer demibranchs are normally shorter than the inner ones but cases where both inners and one outer are eroded have been seen. Such specimens show the uneroded outer demibranch to be the highest but only in the region where the other gills are eroded. The edges of the eroded demibranchs are usually slightly ragged, markedly thickened, and considerably more opaque than the edges of normal oyster gills. As a result of the gill erosions as well as the more rapid growth of the mantle and shell in a ventral direction along with the growth of the crab, an enlarged mantle cavity is produced permitting ample room for the movement of the contained crab. One oyster examined demonstrated the extreme condition which could develop as a result of the presence and activity of a large crab. The oyster was 7.0 cm. long from hinge to bill and 4.8 cm. wide just anterior to the adductor muscle. The periphery of the mantle extended to the edge of the shell in all directions. The distal margin of the demibranchs, however, did not reach the ventral shell margin by 2.1 cm. at the widest part of the oyster. The left demibranchs were only 0.8 cm. high and the eroded right demibranchs only half this size. The fifth stage female crab was 13.0 mm. wide but with outstretched legs and chelae she extended over an area 4 by 2 cm. At first glance after turning back the mantle the body of the oyster appeared to occupy only the dorsal half of the cavity formed by the two shells.

The position of the crab within the oyster was studied in relation to the orientation of the oyster on the bottom. It was found that the crab always faced away from the gill with the normal gills over the crab's carapace (Fig. 23) even when the oyster's position on the bottom made it certain that the crab had remained consistently upside down with relation to the earth's gravitational field. In the laboratory also, there were numerous cases where the crabs remained for days in an upside down position on the oyster's gills. It is this persistent maintenance of position associated with the feeding activities of the crabs which produces the type of erosions seen.

The small-crab type erosion is quite different in appearance and consists of a more local, sharply delimited erosion of one or more demibranchs (Fig. 24). Any single or contiguous demibranchs may be eroded or even all four. The earliest cases show a ragged distal edge, but further attack gradually hollows out a space in which the crab is found. The picture reminds one of a portion cut away or punched out of the edge of the gill although in two cases one of the eroded demibranchs was perforated. The margins of the damaged area are usually thickened by what appears to be accumulations of leucocytes and probably regeneration of damaged tissue. Small-crab type erosions are usually seen at the anterior ends of the demibranchs though, occasionally, typical erosions are observed at any point along the gill. The demibranchs are often eroded more than half way to the base. Very rarely in multiple infestations two areas of damaged gill are seen. Except for one case of eroded palps and several cases with a perforated or damaged mantle area in close approximation to typically eroded gills no other sites of injury to the oyster have been found. In a few cases the mantle instead of being actually perforated was blown up into a "blood blister" or a "pus pocket" as a result of the irritating activity of the crab. The localization of injury is almost conclusive proof that gill destruction is produced by the impingement of the crab's method of feeding on the oyster's food-sorting equipment. It is most likely that the initial break in the marginal furrow by causing food trails to end at the break results in intensified activity by the crab at this spot. Such gill damage is not necessary to but incidental to the mode of feeding of *Pinnotheres*. In the case of the first stage crabs, however, the exoskeleton is hard and unyielding. Certainly this contributes to the production of the early lesions though extension to the large-crab type must occur in later stages of development. Since the adaptation of the crab to the oyster has not yet reached a plane where neither suffers injury the oyster-crab, *Pinnotheres ostreum*, must be considered parasitic on the oyster. It is conceivable that further evolution by shortening the feeding time of the first stage crabs, further decreasing the strength of the exoskeleton, increasing the skill with which the crab obtains the mucus-food strings or confining its feeding to the palp-rejected masses, might result in a relationship less harmful to the oyster. The crab would then be a more efficient parasite in Swellengrebel's (1940) sense of the word.

In small-crab type erosions the gill damage frequently extends down to the base of the gill such that the suprabranchial chamber is directly visible through the most proximal portions of the water tubes. Previous types of damage described involve chiefly a decrease in total gill surface available for feeding. The exposure of the water tubes illustrates another type of malfunction of the oyster's feeding mechanism which has been studied in more detail in the laboratory.

When the oyster's gills are experimentally damaged it can be shown that such injuries produce leaks in the water conduction system of the oyster. Since such a leak backward into the mantle cavity means that water already strained must be rehandled several times a decrease in gill efficiency results the degree of which depends on the extent of the leakage. In a typical experiment a dissecting needle was pushed into the inner left demibranch of a small oyster (No. 1) on the half shell (opened six days before) and forced distad tearing a single plica for a distance of approximately 1.8 mm. from the marginal furrow. When the surrounding plicae were tested with ink suspension[5] some of the latter was seen to escape from the torn plica. A portion of the water being strained by six adjacent plicae was observed to be leaking back into the mantle cavity.

When more extensive V-shaped areas were cut out of a demibranch by scissors the leakage was much greater in volume and water strained from a much larger area was involved. A market-size oyster (No. 2) opened and studied on the half shell will serve as a typical example. Such preparations live as long as six weeks in aquaria, especially if water temperatures are generally below 20° C. A wedge of the outer left demibranch was cut out which measured about 1 cm. across the widest part (along the marginal furrow). At the apex the cut penetrated to a point slightly more than halfway to the gill axis or base. The excision was located just posterior to the anterior third of the demibranch. When ink suspension was placed on this demibranch anterior to the cut practically none escaped in the normal fashion from the cloacal chamber but streamed forcefully out of the openings of the severed water tubes. In consequence, the water strained by more than a third of one demibranch had to be rehandled. Ink placed on the inner left demibranch at the level of the cut on the outer left demibranch also escaped in large part from the cut water tubes of the outer left demibranch. Such a loss of efficiency of parts of two gill leaves must reduce the amount of food an oyster can obtain.

As a result of these findings newly-opened, crab-infested, gill-eroded oysters were studied to see whether such leakages occurred naturally. For reasons to be discussed later; namely, the rapid rate of healing and regeneration of the oyster, good examples were hard to find. One notable case, however, examined in January, clearly demonstrated the existence of such injuries. Water tubes of the two inner demibranchs were exposed in the central portion of a rather extensive small-crab type erosion. When tested on the half shell with ink, both affected demibranchs showed significant leaks. Not only the usual loss from ink placed anteriorly to the lesion but even some placed posteriorly escaped from the damaged area. The latter indicates that some water was moving *anteriorly* in the suprabranchial chamber above the inner right demibranch for a short distance.

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[5] The ink suspension is made by diluting Higgins' Eternal Black Carbon ink with bay water. A dilution of about 1–10 with some subsequent evaporation makes a suspension suitable for use. Placed on the gill of a normal oyster the particle sizes are generally so small that almost all the ink is swept through the ostia into the water tubes. In the intact oyster it appears as a cone-shaped black cloud issuing from either or both of the exhalent chambers on the dorsal side of the oyster.

In another experiment a small oyster on the half shell was placed in a dish with four second stage female crabs. Three days later parts of the demibranchs showed twisted and fused plicae (healed), broken plicae and irregularities of form of filaments not normally seen. One particular area showed extensive damage of nine plicae including destruction of the marginal furrow of eight of these.

To explain the fact that serious leaks are not often naturally found we studied the daily changes in experimentally produced gill lesions of oysters on the half shell. The lesions healed in such a remarkably short period of time that we can easily understand our previous difficulties.

In oyster No. 1, for example, twenty-four hours after the gill injury was produced marked healing had occurred. Three days later (water temperatures in intervening period below 10° C.) much of the damage had been repaired. Anatomically, as well as by ink test, the hole had been plugged by tissue though reorganization of filaments and the marginal furrow had not occurred.

Another small oyster (No. 3) healed much more rapidly. In this case ink escaped from three small experimentally injured areas when tested soon after injury. Less than 24 hours later the holes were sufficiently bridged over by local contractions and regeneration to make water tight plugs.

Another small oyster receiving wedge-shaped excisions of both left demibranchs healed over the exposed water tubes of the shallower one and more than half of the more seriously damaged one in less than a day. Total closure of the wound was accomplished by the fourth day.

Oyster No. 2 likewise healed over though here the excision was so broad and deep that a longer period was necessary for recovery. At the twenty-four interval little microscopically-visible healing could be seen but loss of ink occurred only when placed over a space extending twelve plicae anterior to the excised area. A large amount of ink placed on the inner left demibranch at the level of the injury still showed some loss from the exposed water tubes of the outer left gill leaf.

By the fourth day almost half the exposed openings were bridged over with tissue. On the sixth day ink placed more than two plicae anterior to the edge of the excision no longer escaped from the cut water tubes. When placed proximal to the cut, ink escaped from less than half the originally exposed water tubes and then only at the apex of the wedge where the most exposure occurred. Now, also, only a small amount of the ink placed on the other demibranch (inner left) was lost at the cut surface. By the tenth day microscopic examination and ink tests showed that the gap had been completely closed. Although the damage was not yet all repaired the most serious effects were circumvented. It is reasonable to suspect that the intact oyster under more favorable conditions could respond even more quickly than has been demonstrated in these experiments. Since the crab probably does not confine its activity to a single plica for any considerable length of time a reasonable explanation of the appearance of the gill erosions in recently-opened oysters is at hand.

Gill-damaged oysters lose their crabs at times or eject dead ones. Eventual reconstitution of essential gill structure may then take place though cicatrization and "scar" formation are noticeable. None of the interesting histological aspects of the healing process have yet been studied.

Other Effects of Invasion

Oysters exposed to prolonged storage in air or earth were found to vary in their keeping qualities. It was soon noted that most of the weaker oysters were crab-infested. Whether this was due to the poorer condition of the oyster, irritation by the crab or greater loss of shell liquor due to the enlarged mantle cavity is not known. From the commercial viewpoint, however, this is a serious matter. Much of these losses automatically are circumvented because the oysterman selects for market only his best oysters. It should be emphasized here that even in multiple infestations the presence of more than one gill erosion is extremely rare. An oyster containing fifty crabs is more likely to have no greater eroded area than an oyster containing only a single crab. It is our belief, therefore, that multiple infestations are the result not only of the presence of large numbers of invasive crabs in the water but also of the weakness produced by the activity of a primary crab invasion or any other condition producing weak oysters. The malfunction or even complete stoppage of the feeding mechanism under such conditions may account for the lack of further gill erosions. Such oysters were probably unable to survive the winter and contributed to the heavy mortalities which occurred in some areas after the winter of 1941–1942.

Invasion of Oysters by Crabs

Orton believes that the hard, flat shell of the invasive first stage crab (*P. pisum*) is of value not only in slipping within the valves of the mollusc but in avoiding crushing by the closing valves. We believe this to be largely true for *P. ostreum* and the oyster, though successful invasion does not always occur.

In one experiment an oyster dredged for market was placed in an aquarium with one hundred and fifty first stage *Pinnotheres*. The crabs were scattered over the dish at the start of the trial. Within a short time many were seen lined up around the oyster from hinge to bill mostly at the places where the two valves come together. Seven days after the start only twenty-five live and twenty *dead* crabs were found in the dish but none were now present on the oyster. The oyster was then opened. There were forty-nine live crabs in the left valve on opening, most of these were around the edges of the shell on the incurrent side where the oyster had apparently begun to wall off the crabs. Twenty-six more crabs were found on the ventral side of the oyster, mostly on the gills but *no gill erosions* were present. Sixteen crabs were on the dorsal side, three in the promyal chamber, two deep in the cloacal chamber, three deep in the left suprabranchial, three in the right suprabranchial chamber and two so deep in the water tubes of the anterior end of the inner right demibranch that the gill had to be dissected to free them. The oyster was 108 × 66 mm. in size and contained one hundred and four of the crabs. All but one of the original crabs were found. Two of the other trials with various oysters yielded invasions of seventy-seven of one hundred and eighty-one and forty-nine of one hundred and twenty-nine crabs.

In all trials a few dead crabs were seen and in one series of closely examined oysters exposed to only a few crabs the fate of several was followed. A typical case showed a crab attached to the oyster shell opposite the opening of the cloacal chamber. It had assumed this position within a half hour and almost appeared as though its posterior end were caught within the oyster's shell. Seven hours later it had scarcely moved in position but one hour later it was found on the bottom of the dish on its back, dead, directly below the position it had been occupying on the shell of the oyster. It had apparently attempted entry and partially succeeded but was compressed until dead and then ejected. In another case a weak crab was removed from the bottom of a dish for examination. It had lost parts of two legs on one side. In still another instance two specimens which had effected entry, so far as could be detected, were later ejected from the oyster as dead crabs. These probably had effected almost but not complete entry.

Note on Collecting Specimens for Future Study

In commercial packing of oysters the oysters are drained—in part to insure accurate measurement of the volume of the oysters opened. This volume is the basis of paying the shucker or opener. The oysters are later washed and drained again before shipment. Catching the first drainings in pails and then placing the material collected in shallow pans or dishes of bay water allows the crabs, freed in the opening of the oysters, to be readily recognized and collected.[6] We have been successful in obtaining specimens of all stages of *P. ostreum* in this fashion especially from areas where crab density is very low.

Discussion

It is obvious from the data presented that *Pinnotheres ostreum* can no longer be considered other than a parasite of the oyster. That we witnessed a most unusual sequence of events in 1941 is without question for the dramatic appearance of an oyster containing one hundred or more crabs can hardly be overstated. The subsequent decrease of numbers in 1942 is another example of the population cycles so often seen in nature. We have also seen the abrupt rise and fall in numbers of starfish, *Stylochus ellipticus* and *Polydora ligni* (Nelson and Stauber, 1940), on the oyster grounds in Delaware Bay in recent years. The rise of each was associated with heavy, localized mortalities.

It is not surprising that the evidence gathered here was not previously noted. The small size of the first stage crabs and the need for careful opening and search of oysters in order to detect their presence are prime reasons. For example, we have repeatedly seen cases with gill erosions but could detect no crab on the first examination. Placing these oysters on the half shell in dishes of bay water for a few hours a subsequent examination would often reveal the presence of a crab. Furthermore, among oysters held out of water for a time we have found specimens of first stage *P. ostreum* dried fast to the edge of the oyster's shell as though they had attempted to leave the unfavorable environment and were trapped by lack of an ambient fluid medium in which to move. This being the case it would take such an unprecedented increase in density as occurred in 1941 to focus one's attention on the causal relationship between crab and oyster gill damage. The very fact that in over one hundred years only two specimens of male crabs were obtained by collectors is in itself suggestive. Nor can the fact that oystermen always select for quality of meats be overlooked. Only someone searching for and among poor oysters or investigating causes of mortality would be apt to find the crabs.

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[6] I am indebted to Dr. T. C. Nelson for this fruitful suggestion.

The large crabs, however, have long been known and their very existence means that the earlier stages were also present though undetected. Dean's description of malformed or stunted palps with thickened outgrowths is clearly indicative that the parasitism has not been acquired recently. But the shortening of demibranchs on the affected side of an oyster containing a Stage V crab is not always readily distinguished especially since there are minor differences in the heights of the demibranchs normally. In a significant proportion of the cases also, since the erosions are present on only one or two of the demibranchs, the damaged area is shielded from cursory glance by the unaffected gills which cover them. This is even true of the small-crab type lesion in many cases.

It would be of great interest to learn whether these findings are duplicated at other points along the eastern seaboard where the presence of *Pinnotheres ostreum* within the valves of *Gryphaea virginica* has long been known to exist.

SUMMARY

1. All the parasitic stages of *Pinnotheres ostreum* are described, many for the first time. Besides the invasive first stage male and female crabs, one other male stage and four other female stages are reported.

2. The first crab stage (male or female) is hard-shelled, relatively flat and hairy with a distinctive brown color and white markings.

3. Subsequent stages are quite different with membranaceous, yielding carapace, slender legs, and more rounded body form.

4. The later stages of the females are distinguished from one another by size, color, width and shape of abdomen with relation to the rest of the body and size and differentiation of the pleopods. Maturity is usually reached in the fifth stage female and ovigerous crabs have been seen.

5. All stages of crabs have been found in oysters either singly or in various combinations except that no more than one specimen of a Stage III, IV, or V female has ever been observed within a single oyster.

6. Multiple infestations have been observed which involve chiefly first stage crabs. One oyster contained 262 of these crabs.

7. The oyster crab feeds chiefly on the particulate food material strained from the water by the oyster's food sorting mechanism.

8. In so feeding the crab injures the demibranchs of the oyster causing erosions which interfere with normal feeding of the oyster. These erosions are sharply localized in oysters with first stage crabs.

9. Damage to the oyster gills involves a reduction in the amount of area available for food collecting and in the efficiency of both the straining and the collecting mechanisms. The more serious lesions may also show leakages of the water pumping or conduction system which is such an important feature of food sorting.

10. Rapid healing and regeneration of gill tissue almost keeps pace with destruction and probably saves many oysters from death.

11. The hard shelled, almost tick-like, invasive first stage crabs are less adapted to life within the oyster than the modified later stages. The latter are more resistant to their host's exposure to unfavorable environmental conditions such as storage out of water.

12. Experimental invasion of oysters by crabs has been accomplished but artificial duplication of typical, extensive gill lesions has not yet been obtained.

13. The unprecedented invasion of oysters in 1941 was followed by a lesser invasion in 1942 and probably will follow the usual course of population cycles in future years.

14. The parasitic nature of the crab's relations with the oyster is outlined and reasons suggested why this previously was not found out.

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